Master´s Thesis

The potential of exosome-based gene therapy to eradicate glioblastoma cells

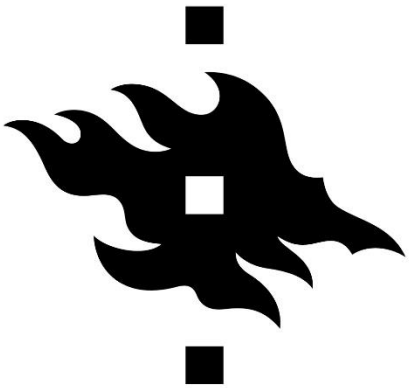
Felicia Bütün

International Master´s program in Translational Medicine

Faculty of Medicine

University of Helsinki

May, 2021



UNIVERSITY OF HELSINKI

Table of Contents

Abstract

New treatment methods are urgently needed for glioblastoma (GBM), the most common malignant primary brain tumor in adults, that currently lacks any curative treatment. Targeted therapeutic approaches have shown promising results already, but common drug delivery vehicles come with efficacy issues and are restricted by their safety and toxicity profiles. Exosomes, cell-produced nanosized vesicles, have emerged as a new potential carrier for gene therapies in cancer treatment due to their natural material transport properties, biocompatibility, and specificity in transporting cargo to the target cells. These extracellular vesicles have the additional advantage of being able to cross the blood-brain-barrier (BBB), which makes them especially valuable for brain malignancies, such as glioblastomas. So far, gene therapy approaches in exosomes have focused on RNA in cancer treatment, but research findings are limited with plasmid-based gene therapies using exosomes. The main concern has been whether the increased plasmid size would decrease the transfection efficiency of the plasmid into the exosomes.

This study aimed at setting-up exosomes as plasmid-based gene therapy nanocarriers. To achieve this, different plasmid-based gene therapies were tested, including the targeting of common aberrations of GBM cells to impair proliferation and the use of cytotoxins to induce apoptosis in the target cells. The plasmids were transfected into exosomes and subsequently inoculated into patient-derived glioblastoma cells with the aim of decreasing the number of glioblastoma cells. The findings of this study demonstrate a successful set-up of an exosome-based gene therapy in patient-derived glioblastoma cells by using engineered HEK293FT cell derived exosomes consisting of a plasmid-based combination gene therapy encoding the cytotoxins Granzyme B and Diphtheria toxin fragment A.

Abbreviations

ADPR	Adenosine diphosphate ribose
AV	Adenovirus
BBB	Blood-brain-barrier
bp	Base pairs
BSL-2	Biosafety Level 2
CAFs	Cancer-associated fibroblast
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CTLs	Cytotoxic T lymphocytes
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded DNA
DT	Diphtheria toxin
DTA	Diphtheria toxin fragment A
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EF2	Elongation factor 2
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
EtOH	Ethanol
FGF	Fibroblast growth factor
GBM	Glioblastoma
GFP	Green fluorescent protein
GzmB	Granzyme B
HDM2	Human double minute 2
HEK	Human embryonic kidney
IDH1	Isocitrate dehydrogenase 1
IL-8	Interleukin 8
IT	Immunotoxins
kb	kilobase
LB medium	Lysogeny broth

MDM2	Mouse double minute 2 homolog
MEM α	Minimum Essential Medium α
MET	Mesenchymal epithelial transition factor
MGMT	O[6]-methylguanine-DNA methyltransferase
miRNA	Micro ribonucleic acid
mQ	MilliQ
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NK	Natural killer
NLuc	NanoLuc
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF-A	Platelet-derived growth factor A
PDGFRA	Platelet-derived growth factor receptor A
PI3K	Phosphoinositide 3-Kinase
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PTEN	Phosphatase and Tensin homolog
RAS	Rat sarcoma
RB1	Retinoblastoma gene
RNA	Ribonucleic acid
SEC	Size Exclusion Chromatography
siRNA	Small interfering ribonucleic acid
TAE	Tris acetate EDTA
TCC	Transitional cell carcinoma
TKR	Tyrosine kinase receptor
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1 Introduction

1.1 Glioblastoma

Glioblastoma, which is classified as a grade IV glioma by the World Health Organization (WHO) and arises from progenitor or neuroglial stem cells (Tang et al., 2019), is the most lethal form of cancer in humans (Mrugala, 2013) and the most severe and common primary tumor within the brain in adults (Wirsching et al., 2016). The World Health Organization classified glioblastoma as a grade IV diffuse astrocytic and oligodendroglial tumor with a yearly incidence of 0.59 to 5 per 100,000 persons (Grech et al., 2020). A slight increase of the incidence in the last 20 years has been noted, which can be explained with an improvement in radiologic diagnosis and with a rising incidence in older patients (Alifieris & Trafalis, 2015).

The incidence for men is increased 1.6 times compared to the incidence for women and the risk for Caucasians has been reported 2.0 times higher than the risk for Afro-Americans and Africans, with Asians and American Indians showing a lower incidence (Tamimi & Juweid, 2017). While glioblastoma can develop at any age (Hanif et al., 2017), the mean age when glioblastoma occurs is 62 years (Grech et al., 2020). The average survival of the glioblastoma patients following diagnosis is 14 to 15 months (Hanif et al., 2017), emphasizing the need for new efficient therapies.

The symptoms of glioblastoma depend on many factors such as the region of the brain that is affected, the tumor size, the location of the tumor and secondary effects, including a rise in intracranial pressure. The symptoms vary from seizures, focal neural deficits, cognitive impairment to changes in behavior, balance issues and headaches, which are especially common and present in 30 – 50% of glioblastoma patients (Hanif et al., 2017).

The research for risk factors in the development of glioblastoma is ongoing but so far solely radiation and certain genetic syndromes were identified as causes for GBM, while environmental factors, including an unhealthy diet, mobile phones, exposure to pesticide or smoking were found not to be associated with glioblastoma (Hanif et al., 2017). As of now, there is no known method of preventing the development of glioblastoma (Gallego, 2015).

The pathogenesis of glioblastoma has been a consistent topic of research to develop new therapies and an understanding of the tumor resistance. This disease is characterized by necrosis, angiogenesis, genomic aberrations, infiltration, uninhibited cell proliferation and apoptosis resistance (Kesari, 2011). Overall, there are two clinical forms that have been described for GBM, the primary and secondary glioblastoma. 95% of patients present with the primary GBM, which arises de novo in a span of 3 to 6 months and is mostly seen in elderly. Meanwhile secondary GBM presents in younger patients and develops from previous low-grade astrocytoma. For both forms, the same pathways are altered and the response to treatment does not differ much. The epidermal growth factor receptor (EGFR) is often amplified and mutated in primary GBM, encoding an altered EGFR, called epidermal growth factor receptor variant III (EGFRvIII), while an increase in signaling through the platelet-derived growth factor A (PDGF-A) receptor has been discovered in secondary GBM. Both mutations result in an increase of the activity of the tyrosine kinase receptors (TKR) and in consequence activates the rat sarcoma (RAS) and phosphoinositide 3-Kinase (PI3K) pathways. The prevalent mutations in primary GBM have been found in the mouse double minute 2 homolog (MDM2) gene, encoding for a p53 inhibitor, the homozygous deletions of cyclin-dependent kinase inhibitor 2A (CDKN2A) and additionally, mutations of phosphatase and tensin homolog (PTEN), whereas p53 mutations, an amplification of mesenchymal epithelial transition factor (MET), platelet-derived growth factor receptor A (PDGFRA) overexpression and mutations of isocitrate dehydrogenase 1 (IDH1) have been connected to secondary GBM. Furthermore, a rise in activity of human double minute 2 (HDM2) and the inactivation of the retinoblastoma gene (RB1) have shown an association to low-grade gliomas progressing to high-grade gliomas and result in altered signaling pathways, which are mediated by growth factors and a change in cell cycle regulation. This results in apoptosis inhibition, angiogenesis, invasion and an increase in cell proliferation (Alifieris & Trafalis, 2015).

The diagnosis of glioblastoma is usually done by a magnetic resonance imaging (MRI) scan, which is followed by a biopsy of brain tissue. These techniques are performed if patients present with symptoms that raise suspicion, such as headaches, nausea, cognitive impairment and motor weakness (Batash et al., 2017).

Worldwide, there has been a considerable amount of research for the treatment of glioblastoma (Mrugala, 2013) and yet the prognosis for GBM remains poor with the

majority of treatments being palliative (Kesari, 2011). Even though molecular pathways responsible for the invasive nature of glioma continue to be found, GBM is still named as one of the major challenges in clinical oncology, which is also due to the slow translation of the basic research in the laboratory to the clinic and the patients. Over the recent years, varying methods of treating glioblastoma have been studied with little success. The reasons for this include the heterogeneous biology and a diffuse nature of the GBM growth (Mrugala, 2013). Currently, the standard of care of glioblastoma is defined by a multimodal approach, which consists of radiation, chemotherapy and surgical resection (M. E. Davis, 2016). The concept of treating GBM for newly diagnosed patients consists of maximal surgical resection of the tumor prior to radiation therapy with simultaneous systemic chemotherapy utilizing the alkylating compound temozolomide (Mrugala, 2013), which is the norm for the first-line treatment (Gallego, 2015). Patients with a methylated O[6]-methylguanine-DNA methyltransferase (MGMT) promoter have been connected to an increase in the sensitivity to the drug temozolomide, resulting in longer survival compared to the patients with an unmethylated MGMT promoter. The methylation status of the promoter can be tested by performing a DNA test on the glioblastoma cells (Martinez et al., 2007). Additional treatment consists of treating symptoms such as cerebral edema, fatigue and infections (Alifieris & Trafalis, 2015). One of the defining issues of the GBM treatment is that not all the tumor cells will be removed due to the highly invasive nature of glioblastoma cells, which spread over white matter and blood vessels. Therefore, a complete resection is not possible. Additionally, the self-renewal of the tumor cells and the resistance to radiation and chemotherapy is benefited by a disseminating tumor stem-cell like cell population, resulting in the recurrent nature of glioblastoma and overall a worse prognosis for long-term survival (Kwiatkowska et al., 2013). Standard treatment options, such as surgery are often inefficient since the tumor is scattered and cannot be removed completely without harming the brain. In addition, patients with tumors in the basal ganglia, brain stem or eloquent cortex are not responsive to surgery and consequently display a worse prognosis. Chemotherapy treatment is connected to certain limitations as well. Mostly because of numerous chemotherapeutics not being able to get past the blood-brain barrier, resulting in serious diminishment of the drug delivery to the parenchyma of the brain and the tumor. Additionally, glioblastoma shows resistance to chemotherapy due to a hypoxic tumor environment. Due to all these reasons, it is not possible to have a universal treatment approach for all

glioblastoma patients, but instead multi-targeted therapy is needed (Mrugala, 2013), with the treatment being dependent on the onset of the disease, the patient age, the time of diagnosis and if the tumor is recurrent (Alifieris & Trafalis, 2015). The progress in surgical intervention, chemotherapy and radiotherapy improve the survival of patients with glioblastoma and contribute to a higher quality of life (Mrugala, 2013), but so far, the disease will recur in the large majority of patients (Gallego, 2015).

Overall, glioblastoma is one of the human cancers that show the worst 5-year survival rates of 4-5% and solely 26-33% of the patients survive beyond two years in clinical trials, proving once more that the current treatments only serve as an extension of the overall survival and not as a cure for glioblastoma patients. Present treatment limitations, GBM´s diffuse nature, advanced age of onset and a limited comprehension of the pathophysiology of glioblastoma can be named as reasons for the general brief survival span (Batash et al., 2017). However, there is hope for new treatment methods, including targeted therapies, which became available due to the discoveries in the recent years about the tumor biology of glioblastoma (Kesari, 2011).

1.2 Gene therapy in glioblastoma cells

With the aim of prolonging overall survival of glioblastoma patients and the limited treatment options that are available at this point, it is obvious that new efficient therapy approaches are urgently needed as an alternative or complement to present treatment plans. Gene therapy, which molecularly targets the tumor and its surrounding microenvironment has emerged as a potential new treatment for glioblastoma. While gene therapy has originally been designed to mostly improve and treat genetic diseases that occurred due to the deficiency of a single gene, the main approach for gene therapy in cancer has been focused on delivering therapeutic genetic material to the tumor cells in order to eradicate cancer cells or alternatively amend the response of the immune system against the cancer cells (Kwiatkowska et al., 2013)

So far gene therapy has been focused on mRNA-based therapeutic models, advantages of which consist of fast and temporary expression, flexible convertibility, and simple manipulation. All these advantages make it possible to handle the variable and complex nature of glioblastoma and thus synthetic anticancer mRNAs taken up by diverse delivery vehicles have been shown to succeed in crossing biological barriers and attacking the tumor cells (Tang et al., 2019).

Several main gene therapy approaches have been developed in the past years, with the main ones focusing on the delivery of cytokine genes to enhance the immune response, the delivery of suicide genes with the aim of apoptosis induction in the tumor cells, the delivery of viruses, that show a conditional replication and result in the lysis of cancer cells without harming healthy tissue and the transfer of tumor-suppressor genes to tumor cells, which leads to reprogramming of the cancer cells into programmed cell death. Many of these strategies have shown success in pre-clinical models and in recent years, a constant rise in the amount of gene therapy models in clinical trials could be noted (Kwiatkowska et al., 2013). However, delivery of the therapeutic genes into cancer cells could only be detected within a close distance from the delivery site, which has been one of the limiting factors of success in clinical trials (Clarke et al., 2010). Viruses have been shown to be the main delivery vehicles for the gene therapy but there are alternative carriers, including nanoparticles, liposomes, and stem cells, which can be utilized for the delivery of the genetic material to the cancer cells (Kwiatkowska et al., 2013).

1.2.1 Targeting plasmids for common genetic alterations in glioblastoma cells

Cancer cells derive from normal cells by developing mutations in key genes, such as tumor suppressor genes or oncogenes (G. Castro et al., 2011). Tumorigenesis in humans develops over multiple steps with each step involving genetic alterations driving the continuous transformation of healthy cells into malignant derivatives (Hanahan & Weinberg, 2000). The altered genes are responsible for important cellular processes, including apoptosis and proliferation. Numerous mutations in genes in charge of angiogenesis, cell cycle progression, rising motility and growth factor independence need to accumulate to result in tumorigenesis. The main characteristics of gliomagenesis are known and consequently gene therapy has been developed with a focus on repairing common genetic aberrations in glioblastoma cells (G. Castro et al., 2011).

1.2.1.1 Inhibition of oncogene EGFRvIII

Epidermal growth factor receptor has been shown to promote growth of glioblastoma cells by activating the intracellular tyrosine kinase domain through the binding of its ligand, epidermal growth factor (EGF) to the extracellular part to initiate various

signaling cascades. The signaling activity of EGFR, which is frequently caused by mutations, such as EGFRvIII, is often abnormally elevated in glioblastoma cells. This specific mutation is caused by an in-frame deletion of exons 2 to 7 and can exist without an amplification of EGFR (Padfield et al., 2015). This deletion in the extracellular binding domain, which has been shown to be part of approximately 40% of GBMs, leads to a constitutively active tyrosine kinase domain (Clarke et al., 2010). EGFRvIII is the most important variant among the mutations of EGFR (Bălașa et al., 2020) and is connected with an increase in the proliferation of glioma cells (Montano et al., 2011) and results in resistance to chemotherapy and apoptotic stimuli (Bălașa et al., 2020). Consequently, the primary part of my MSc thesis project´s gene therapy approach includes a plasmid containing inhibitory RNA molecules, downregulating the expression of the EGFRvIII target gene.

1.2.1.2 Overexpression of tumor suppressor gene TP53

The tumor suppressor gene TP53 is the guardian of the genome and its main function includes detecting genetic abnormalities, that occur in the process of DNA synthesis. Following the detection of a genetic abnormality, the cell cycle progression is stopped, and the repair process is observed. In case of a significant damage to the DNA, apoptosis is induced by the p53 protein encoded by the TP53 gene. Quiescent cells do not show any p53 expression, yet expression is seen in cells undergoing cell cycle progression and as a result of DNA damages (G. Castro et al., 2011). Consequently, this tumor suppressor gene is a vital part of the reduction of tumor development and it is either carrying mutations or missing in more than half of human tumors, while mutations in parts of the p53 pathway have been detected in 90% of all human tumors (G. Castro et al., 2011). Additionally, in glioblastoma, deregulated parts of the p53 pathway have been suspected as part of cancer cell stemness, proliferation, cell invasion, migration, proliferation, and apoptosis evasion. Overall, TP53 is among the most frequently deregulated genes in cancer cells and a deregulation of the p53 pathway occurs in 94 percent of GBM cell lines and in more than 4 out of 5 patients with glioblastoma (Y. Zhang et al., 2018). Consequently, the first part of the gene therapy in this research project focuses on overexpressing the TP53 gene in order to support tumor suppression in glioblastoma cells.

1.2.1.3 Overexpression of tumor suppressor gene PTEN

PTEN (phosphatase and tensin homolog) is defined as a phosphatidylinositol-3,4,5-trisphosphate (PIP3) phosphatase and plays a crucial role in tumor suppression by antagonizing the oncogenic PI3-kinase signaling pathway (Yang et al., 2017). The PI3K/AKT/mTOR pathway is part of the primary molecular pathways responsible for the expansion of glioblastoma cells and normally inhibited by PTEN and thus a mutation of PTEN promotes severe proliferation of the cancer cells (Bălașa et al., 2020). In glioblastoma, the tumor suppressor gene PTEN is frequently mutated or deleted, which results in a resistance to therapy. Moreover, it has been shown that mutations that result in reduced protein function in PTEN are associated with a deficient response to chemotherapy and radiation therapy treatment in brain cancer patients and in addition display a connection to metastasis. This demonstrates the role of PTEN as a central regulator of tumor sensitivity to a variety of treatments (Benitez et al., 2017). Therefore, overexpression of PTEN is part of the first approach of the plasmid-based gene therapy, that has been conducted in this project.

1.2.2 Targeting plasmids utilizing apoptosis-inducing Granzyme B enzyme

Targeted therapy could avoid the disadvantages that often come with conventional treatments in cancer patients, such as an insufficient specificity and serious side effects. One of the approaches in targeted therapy consists of immunotoxins (ITs), which include cell-binding and apoptosis-inducing components delivered directly to malignant cells. Granzyme B is an enzyme that has proven to be a promising candidate for targeted therapy in cancer cells (Hehmann-Titt et al., 2013).

Granzyme B (GzmB) is defined as a serine protease and part of secretory granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. CTLs and NK cells utilize granzyme B to induce apoptosis as their primary mechanism of action to eradicate targets such as cancer cells. Granzyme B and additional granule proteins such as perforin are secreted into the immunological synapse by the engaged natural killer cells and cytotoxic T lymphocytes following the detection of the target cells. Subsequently, Granzyme B is translocated into the target cell´s cytoplasm, in which several protein substrates are cleaved and activated or inactivated by the protein, leading to the target cell´s apoptosis (Rousalova & Krepela, 2010).

In this research project, Granzyme B was selected as one of the potential candidates for the plasmid-based gene therapy to induce apoptosis in glioblastoma cells.

1.2.3 Targeting plasmid utilizing apoptosis-inducing Diphtheria toxin fragment A

Diphtheria toxin (DT) is one of the main cytolethal toxins used in the construction of immunotoxins targeted cancer therapy (Chandramohan et al., 2012) focusing on gene delivery and cell-specific toxin expression (Shafiee et al., 2019). One molecule of toxin will result in an irreversible inactivation of 300 ribosomes in 35 minutes, thereby efficiently killing tumor cells (Chandramohan et al., 2012). Diphtheria toxin, a single chain 62 kDa protein, includes 535 amino acid residues and inhibits protein synthesis in susceptible cells, thereby being cytotoxic (Shafiee et al., 2019). The toxin consists of two regions, the 21-kDa fragment A (J. Zhang et al., 2012), located at the N-terminus contains a catalytic domain, and is responsible for halting the protein synthesis in eukaryotic cells (Shafiee et al., 2019), by inactivating the elongation factor 2 (EF2) (J. Zhang et al., 2012). The second fragment defined as fragment B, is located at the C-terminus and includes a transmembrane domain and a domain for receptor binding (Shafiee et al., 2019). The 37-kDa fragment B is responsible for delivering the A fragment (DTA) into cells by attaching to receptors on the cell surface (J. Zhang et al., 2012). The protein synthesis is inhibited by the binding of the catalytic domain to nicotinamide adenine dinucleotide (NAD) in the cell´s cytoplasm, followed by translocation of an adenosine diphosphate ribosyl (ADPR) moiety to EF2, hereby inactivating it (Shafiee et al., 2019). The DTA protein is highly toxic and has been commonly used in targeted tumor therapy to eradicate cancer cells (J. Zhang et al., 2012).

Due to its toxicity and proven efficiency as cancer therapy, Diphtheria toxin fragment A was selected as a potential candidate for the project´s plasmid-based gene therapy to induce apoptosis in glioblastoma cells.

1.3 Exosomes as potential new delivery vehicle for gene therapy

So far, gene therapy has focused mostly on plasmids, viral vectors, cosmids and artificial chromosomes as the four major vector types. However, those options suffer from some disadvantages. Viral vectors are connected with serious safety concerns,

while the production of non-viral DNA vectors is limited by a low transfection efficiency and a potential mutation induction (Tang et al., 2019).

In recent years, exosomes have come into the research focus as potential tumor targeted vehicles to transport therapeutic molecules directly to tumor cells. Exosomes are secreted and subsequently naturally taken up by cells and have shown the ability to steadily translocate pharmaceutical compounds, proteins, and therapeutic microRNAs. Since the cargo of exosomes includes genetic material, this can be taken advantage of by developing exosomal therapy utilizing microRNAs. MicroRNAs (miRNAs) are small-sized, non-coding RNAs, known to bind to the 3´-untranslated region of mRNAs and subsequently silencing or degrading the target RNA, hereby inhibiting translation and thereby the protein synthesis (Gilligan & Dwyer, 2017). In the treatment of breast, pancreatic, prostate, lung cancer and glioblastoma, exosomes have been used for the delivery of conventional chemotherapeutics, natural products and RNA (Pullan et al., 2019).

While so far, the focus has been on small interfering RNA (siRNA) and miRNA in exosome-based gene therapies for the treatment of varying diseases (Orefice, 2020), this research project focuses on using an exosome-based gene therapy, which utilizes a plasmid-based therapeutic model. So far, no in-depth research is available for the use of exogenous plasmid DNA in extracellular vesicles (Orefice, 2020).

1.3.1 Characteristics of exosomes

Exosomes are defined as a subtype of extracellular vesicles, which are released by varying cell types and contain components such as proteins, RNA, lipids, metabolites, or DNA. They can be taken up by other cells, potentially leading to changes in cell behavior and function (Kalluri & LeBleu, 2020). Exosomes originate from multivesicular bodies, which are defined as intraluminal vesicles of late endosomal compartments and these endosomes fuse with the cell membrane, thereby allowing the vesicles to be released into the extracellular compartment. In addition, extracellular stimuli or certain elements, resulting in stress, may positively influence their generation (De Toro et al., 2015). The nanosized extracellular vesicles are part of intercellular communication in normal as well as in pathological conditions (Sharma et al., 2016) and do so by trafficking molecules between cells (Srivastava et al., 2015). Moreover, exosomes contain cell-of-origin biomacromolecules and thus reflect the cell's

molecular bioprint (Sharma et al., 2016). In addition, exosomes are known to contain active genetic information in their lumen such as DNA, miRNA, mRNA and active peptides, which are characteristic for the parental cell and various fluids in the human body can be utilized for the isolation of these genetic components. Due to the ability of exosomes to translocate components from one cell to another while being able to cross biological barriers such as the blood-brain barrier, without triggering the immune system, these extracellular vesicles have been developed as novel acellular carriers for pharmaceutical compounds (Srivastava et al., 2015).

1.3.2 Exosomes in cancer – friend or foe?

Exosomes play a dual role in cancer therapy. On one side, the extracellular vesicles have been associated to varying hallmarks of cancer by impacting neoplasia, metastasis, the growth of tumors and resistance to several chemotherapeutic agents and antibodies (Kalluri & LeBleu, 2020). In the essential step of growth and spreading of the tumor, exosomes have been associated with the tumor angiogenesis and extracellular matrix transformation (Kalluri & LeBleu, 2020). Additional research has shown that exosomes play a crucial role in metastasis by travelling to distant organs and promoting the formation of a pre-metastatic niche by interacting with the stromal cells (Bastos et al., 2018). Further, exosomes are able to interact directly with therapies, hereby reducing their efficiency against the tumor cells. In addition, exosomes, secreted from cancer-associated fibroblasts (CAFs) are capable of altering the cancer cell´s transcriptome to support the tumor cell´s survival (Kalluri & LeBleu, 2020). Moreover, exosomes secreted from cancer cells contain an elevated amount of vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8), which promotes the induction of neovascularization, a process essential for the formation of vessels. The invasion of tumors is facilitated by cancer exosome's ability to support matrix remodeling and enhance the expression of matrix metalloproteinase in fibroblasts. Furthermore, the extracellular vesicles promote an environment, which is prone to the development of tumors due to the promotion of the immune escape of tumor cells by regulating the activity of immune cells. These findings stress the importance of studying the potential of targeting the exosome-mediated communication as a treatment approach in cancer therapy. Another research focus has been the targeting of

exosomes with the aim of blocking their support function in the progression of tumors and therapy resistance (Bastos et al., 2018).

However, exosomes have shown promising potential in improving cancer therapy as well, by being applicable as carriers of anti-cancer molecules due to their stability in circulation, their ability to directly target tumors and their intrinsic capability of horizontally transferring cargo. In cancer and other diseases, exosomes are investigated as new delivery vehicles for drugs due to their natural presence in body fluids and their biocompatibility and biodegradable nature. As a consequence of this, exosomes have been shown to have reduced toxicity and to be less immunogenic when compared to other nanocarriers. Overall, exosomes show promising potential to enhance the efficiency of cancer treatment by functioning as delivery vehicles for therapeutic drugs, antibodies or RNA for the manipulation of gene expression (Bastos et al., 2018).

1.3.3 Disadvantages of current standard delivery vehicles

Gene therapy has become a promising field in the development of cancer treatment. The common vectors in gene therapy can be divided into viral and non-viral vectors. Though until now, there are several restricting factors responsible for limited success of gene therapies in treating cancer. So far, issues such as limited transfection efficiency of the gene, the precise delivery of the target gene to the target site and the avoidance of toxicity and severe side effects have to be addressed to increase the performance of gene therapies in cancer treatment (Sun et al., 2014). While viral vectors have emerged as the main delivery vehicle of DNA and RNA in gene therapy on account of their transduction efficiency, several problems have appeared. These include serious immune responses and the issue of adenoviral (AV) vectors, which following systemic administration, remain in the liver, thereby compromising the efficiency of the transgene transduction, resulting in hepatotoxicity and death (Orefice, 2020).

Overall, current delivery vehicles are restricted by safety and toxicity concerns, and limited efficiency.

1.3.4 Advantages of plasmid-based gene therapy and exosomal carriers

Plasmid-based gene therapies are associated with several advantages, which make them interesting for cancer therapy when compared to the RNA-based gene therapies. Due to the plasmids modular composition, straightforward molecular cloning is possible, resulting in many potential actions, such as manipulation and design of plasmids for new therapeutic approaches (Hardee et al., 2017). By being able to perform molecular cloning to design the plasmids, it further encourages finetuning and enhancing steps, which can be carried out to increase the targeting efficiency of the plasmid-based gene therapy. In addition, conditional expression, which is defined by specifically expressing the gene therapy in a certain type of cell or certain time, becomes an option. This project uses mir21 and IL13RA2 promoters, to enhance the specificity of the therapy and further reduce cytotoxic effects in case the exosomes target healthy cells. The plasmid-based gene therapy will mostly be active in cancer cells, since the expression of Mir21 and IL13RA2 is substantially upregulated in cancer cells, compared to the healthy cells.

Exosomes are defined as natural cell-produced nanosized delivery vesicle and have been shown to be immunologically inert when isolated from compatible cells and further display an inherent capacity to pass inflexible barriers in the human body (O'Loughlin et al., 2012), making them a novel promising delivery vehicle for gene therapy.

In this therapeutic set-up, exosomes were chosen as the delivery vehicles for gene therapy due to several reasons. These include their numerous advantages as carriers, due to their properties, such as their physical characteristics, including biocompatibility, permeability, minor toxicity, low immunogenicity, and stability. Thus, exosomes are an exceptional material platform, which contains all the features indispensable for successful outcome of any nanoparticle drug delivery system (Pullan et al., 2019). In detail, exosomes have been shown to be steady carriers of therapeutic agents, demonstrated the ability of potential modifications to enhance cell specific homing and deliver therapies directly into the target cells by fusing with the cell´s plasma membrane. Allogenic exosomes, which are derived from mesenchymal stem cells, potentially resolve the major challenge of cell-based therapies by enabling reduction in the immune response. Due to their small size, exosomes can pass barriers, that are not possible to cross for cells (Gilligan & Dwyer, 2017). Furthermore, exosomes show great promise as carriers for various components due to their ability to translocate

natural material and the intrinsic potential for long-term circulation, which is ideal for transporting a wide range of components, such as DNA, RNA, proteins and therapeutic agents (Liu & Su, 2019). Another reason why exosomes have been studied as natural vehicles for the delivery of drugs is due to their ability to move safely in extracellular fluids and efficiently and specifically carry cargo to destined cells (Li et al., 2019). Exosomes further carry the advantage of being able to house lipophilic as well as hydrophobic drugs due to their lipid bilayer membrane holding an aqueous core (Gourlay et al., 2017). Moreover, the efficiency of exosome-mediated strategies has been shown by the first proof-of-concept studies and in a variety of clinical trials in which exosomes have been well-tolerated by the patients, even after prolonged administration. In addition, clinical trials for cancer patients demonstrate the safety and promise of wide-ranging therapeutic implementations that may benefit from this nanotechnology (O'Loughlin et al., 2012).

Overall, exosomes have opened the door to many opportunities in the therapeutic setting due to the possibility of engineering the content of exosomes and their migratory itinerary as well as the relatively simple method of isolating exosomes (Gilligan & Dwyer, 2017). In addition, the plasmid-based gene therapies allow the regulation and finetuning of the plasmid expression in the target cells.

1.3.5 Exosomes in glioblastoma

Glioblastoma cells have been shown to secrete multiple exosomes, carrying components, which have the potential to enhance the tolerance of the immune system towards the tumor cells, stimulate chemotherapy resistance, increase the vascular supply in glioblastoma, encourage the development from healthy cells to tumor cells, and aid in the migration of the cancer cells and the subsequent invasion (Bălașa et al., 2020), proving that exosomes play a crucial role in the progression of GBM tumors.

However, exosomes are a new approach in cancer treatment and because of their ability to pass the blood-brain-barrier, they are a potential new way of treating malignancies in the brain. Compared to liposomes of equal size, these extracellular vesicles have tenfold increased capacity to target cancer cells. This may be due to a specific interaction of the ligand and receptor on the target cell or enhanced endocytosis mechanism. Additionally, the production and engineering of exosomes

can be done *in vitro* with the possibility of adding specific ligands, resulting in an enhanced targeting of tumor cells (Bastos et al., 2018).

Interestingly, radiation therapy could be utilized as a pre-treatment for enhancing the exosome-based therapy uptake in cancer treatment, since radiotherapy has been shown to increase the amount of cancer exosomes, secreted by the cells and their microenvironment (Bălașa et al., 2020). In glioblastoma, radiation treatment, results in a rise of the exosome abundance, secreted by GBM cells and the surrounding normal astrocytes. In addition, due to the radiotherapy, the extracellular vesicles have been shown to be taken up by acceptor cells more easily (Gourlay et al., 2017).

In this current time of molecular modification, exosomes display their worth as instruments to target glioblastoma cells as part of therapeutic strategies in addition to being used as markers for diagnosis and prognosis in cancer (Bălașa et al., 2020).

1.3.6 Exosome-based gene therapy in glioblastoma cells

Expeditious developments in cancer science, involving the discovery of cellular processes and surface antigens that are specific to cancer, have made it possible to combine rational drug design and cancer biology to create new targeted therapeutic approaches (Shafiee et al., 2019). In the past few years, exosomes have become valuable potential instruments to combat the aggressive nature of glioblastoma on account of their structure and ability to transport various cargo, with the aim of producing individual therapeutic approaches that specifically target the cancer cells (Bălașa et al., 2020). Recent studies have shown that exosomes, secreted by brain endothelial cells succeeded in delivering pharmacological drugs across the blood-brain-barrier followed by a decrease of tumor progression. Meanwhile, the drugs, administered without exosome vectors failed in crossing the blood-brain-barrier and stayed within the vascular circulation. These finding confirm that exosome-based therapy opens the door to new possibilities for the treatment of brain tumors by succeeding in delivering anti-tumor agents across the blood-brain barrier, a barrier which so far has been mostly impenetrable for the majority of chemotherapy agents (Gourlay et al., 2017).

Overall, it can be seen that exosomes are breaking down the barriers that have been set with most of the polymeric drug delivery systems, proving to be viable carriers with only minimal toxicity issues and verified biocompatibility (Gourlay et al., 2017).

1.3.6.1 Selection of HEK293FT cell line as exosome source

So far, many studies have focused on exosomes secreted by tumor cells in cancer therapy. However, there are safety concerns due to the role of exosomes in cancer progression and possibility of potential problematic effects as a consequence of a gene therapy utilizing tumor-derived exosomes (Gilligan & Dwyer, 2017).

Therefore, exosomes secreted by the HEK293FT cells were selected for this project. HEK293FT cells were chosen as the exosome source due to high productivity of the biogenesis and secretion of exosomes. In addition, HEK293FT exosomes are very commonly available cells (Lin et al., 2018).

1.3.6.2 Selection of virus transfected OP9 cells as alternative exosome source

The murine mesenchymal progenitor cell line OP9 was used as an alternative source of exosomes since exosomes derived from mesenchymal stem cells have shown potential as novel instruments in basic research with an indicated effect on the targeted delivery of microRNAs in cancer treatment (Wei et al., 2021).

For this purpose, the cells were transduced with a lentivirus, resulting in exosomes, which are expressing the homing peptide iRGD. This homing peptide is known for targeting and penetrating tumors by binding to αv integrins and neuropilin-1 receptors, expression of which is often elevated on the surface of cancer cells (Yin et al., 2017). The tumor targeting peptide works through the RGD motif facilitating the binding to αv integrins on the endothelium of the tumor. Subsequently, neuropilin-1 binds to a motif exposed due to a proteolytic cleavage, resulting in tissue and cell penetration. The homing peptide iRGD thereby increases the activity of drugs targeting the tumor (Sugahara et al., 2009). Consequently, iRGD allows delivery of anticancer drugs to the tumor parenchyma. Usually there is either a chemical linkage of the peptide and the anti-tumor drug or the peptide and the therapy are co-injected (Yin et al., 2017).

1.4 Aim of the project

The aim of this research project was to set up an exosome-based gene therapy in patient-derived glioblastoma cells utilizing exosomes isolated either from the HEK293FT or from virus transfected OP9 cells. Three different gene therapy approaches were aimed to be tested to decrease the cell proliferation of the target cells. The first one targets common alterations in glioblastoma cells, while the other gene therapy approaches focus on using cytotoxins with the goal of inducing apoptosis in the cancer cells. While, the mRNA-based gene therapies have shown great potential in glioblastoma treatment (Tang et al., 2019), this study focuses on plasmid-based gene therapy. Our thinking is that a clear advantage of the plasmid-based therapy is that plasmid DNA is an independent, circular vector, encoding for genes of interest (Orefice, 2020), thereby allowing finetuning of the expression in the glioblastoma cells. Currently, the use of plasmids in exosome-based therapies is an ongoing discussion topic in research due to the limited size of exosomes, which makes it questionable if exosomes manage to take up the plasmids (Orefice, 2020). This issue will be tried to solve through reduction of the plasmid size by cloning the plasmids into miniplasmids, followed by a chemical transfection into the exosomes. This approach is supported by studies, which showed a correlation between a decrease in plasmid size and an improvement in the transfection efficiency (Hardee et al., 2017).

Setting up a successful plasmid-based gene therapy in exosomes would present a new way of controlling gene expression in glioblastoma cells using exosomes as delivery vehicles, thereby leading the way towards the generation of exosome-based gene therapy and new hope for glioblastoma patients.

2 Material and Methods

2.1 Molecular cloning & bacterial methods

2.1.1 Gibson Assembly

The Gibson Assembly Master mix (New England Biolabs 2021, Gibson Assembly®) combines 5´exonuclease, DNA ligase and DNA polymerase in a reaction carried out in a single tube. First the 5´ exonuclease leads to an opening of the complementary sequences for annealing by removing the 5´end sequences. The DNA polymerase then synthesizes DNA, to fill the gaps of the annealed single strand regions and in the last step, the nick is sealed and a link between the DNA fragments is formed. The Gibson Assembly method was used for the cloning of the plasmid constructs. The reaction including the following components is performed on ice. The composition of the Gibson Assembly reaction is displayed in Table 1.

Table 1. Composition of the Gibson Assembly reaction (New England Biolabs 2021, Gibson Assembly®).

	2 – 3 Fragment Assembly
Total Amount of Fragments	0.02 – 0.5 pmols*X µl
Gibson Assembly Master Mix (2X), (New England Biolabs, E2611)	10 µl
Deionized H_2O	10 – X µl
Total Volume	20 µl

The samples were incubated at 50 degree Celsius in a thermocycler (MJ Research PTC-200 Thermal Cycler) for 15 minutes and stored on ice for the following heat-shock transformation.

2.1.2 Polymerase Chain reaction (PCR)

To perform a colony PCR, PCR tubes containing 50 µl of mQH_2O were inoculated with colonies from the corresponding bacterial plate. Afterwards, the colonies were lysed at

100 degree Celsius for 10 minutes in a thermocycler (MJ Research PTC-200 Thermal Cycler) before being centrifuged for 25 seconds at 14000 g (Minispin Plus G, Eppendorf). For the PCR reaction, a Master mix containing mQH_2O, buffer (Thermo Fisher Scientific, F530S), dNTP (BioNordika, BN-1006-08), forward and reverse primer (Sigma-Aldrich) and the Phusion DNA Polymerase (Thermo Fisher Scientific, F530S) was prepared. For a colony PCR the lysed colonies were used as the DNA template, while otherwise a plasmid was used as the DNA template. The composition of the PCR reaction is shown in Table 2. The PCR reaction was carried out in a thermocycler (MJ Research PTC-200 Thermal Cycler), for which the annealing temperature and the elongation time of the program were adapted accordingly. The steps of the PCR program are displayed in Table 3.

Table 2. Composition of the Polymerase Chain Reaction (1x reaction).

Reagent	Volume of Components [µL] per reaction
ddH_2O	x
dNTP Mix (10 mM each), (BioNordika, BN-1006-08)	1
5x Phusion HF buffer (Thermo Fisher Scientific, F530S)	10
Forward primer (Sigma-Aldrich)	0.25
Reverse primer (Sigma-Aldrich)	0.25
2 U/µL Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, F530S)	0.25
Total volume	50

Table 3. PCR Program (30 circles of Denaturation, Annealing & Elongation).

PCR Step	Temperature [°C]	Time [s]
Initialization	10	60
Initial Denaturation	98	60
Denaturation	98	30
Annealing	Template-dependent	30
Elongation	72	30 sec/kb
Final Elongation	72	600
Final Hold	10	Unlimited time

2.1.3 Agarose gel electrophoresis

The Agarose gel was done by preparing 0.01 g/ml Agarose powder (Bioline, BIO-41025) in 1x Tris acetate EDTA (TAE) solution in a microwaveable bottle. The 1x TAE solution was prepared by diluting a 50x TAE stock solution (Mixture of 242 g of Tris base, 57.1 mL of acetic acid, 100 mL of 0.5 M EDTA, pH 8.0) with ddH_2O. The mixture was microwaved for 1 minute until the agarose was entirely dissolved. The agarose solution was cooled down to about 50 degree Celsius and SYBR® Safe DNA gel stain (Invitrogen, S33102) was added in a 1:10,000 ratio and mixed before the agarose gel was poured into the prepared Sub-Cell GT Horizontal Electrophoresis Cell (Bio-Rad, 1704402). After the gel was solidified, the electrophoresis cell was filled with 1x TAE solution until the gel was covered. In the next step, the DNA samples were mixed with 6x DNA Loading Dye (Thermo Scientific™, SM1334) and loaded into the wells of the gel. The molecular weight ladder (GeneRuler 1 kb Plus DNA Ladder, Thermo Scientific™, SM1334) was added to one well before the gel was run at about 100 V. The resulting gel bands were imaged by using the gel documentation system NuGenius (Syngene).

2.1.4 Gel extraction

The extraction of DNA from an agarose gel was performed according to the protocol of the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, 740609.50S).

2.1.5 Ligation

The ligation was carried out according to the manufacturer´s instruction of the NEBuilder HiFi DNA Assembly Reaction Protocol (New England Biolabs 2021, NEBuilder HiFi DNA Assembly Reaction Protocol). The reaction was set up on ice and the reaction components are shown in Table 4.

Table 4. Composition of the DNA Assembly reaction (New England Biolabs 2021, NEBuilder HiFi DNA Assembly Reaction Protocol).

Reagent	2 – 3 Fragment Assembly
Recommended DNA Molar Ratio	vector:insert = 1:2
Total Amount of Fragments	0.03 – 0.2 pmols*X µl
NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, E2621S)	10 µl
Deionized H_2O	10 – X µl
Total Volume	20 µl

The samples were incubated in a thermocycler (MJ Research PTC-200 Thermal Cycler) at 50 degree Celsius for 15 minutes and subsequently stored on ice for the following heat-shock transformation.

2.1.6 Preparation of bacterial plates

The solid LB-agar (Pronadisa, E00400) was slowly dissolved in the microwave before being transferred to a water bath (Julabo® water baths, Millipore Sigma, Z615471) with a set temperature of 50 degree Celsius. Once the temperature of the bottle dropped to 50 degree Celsius, 75 µg/mL of the antibiotic Carbenicillin (Gibco™, 10177012) was added and the LB-agar was poured into 10 cm cell culture dishes. After drying, the plates were stored at 4 degree Celsius for further use.

2.1.7 Heat-shock transformation

The competent DH5-α *Escherichia coli* cells (Thermo Fisher Scientific) were melted on ice for 30 minutes before 50 µl of the bacteria were mixed with the plasmid DNA of interest. After an incubation period of 30 minutes on ice, the mixture was given a heat-shock at 42 degree Celsius for 45 seconds by using a heat block (Thermomixer comfort, Eppendorf). Then the mix was incubated for 5 minutes on ice and 250 µl of Lysogeny broth (LB) medium (HUS-Lab, T00053) were added followed by an incubation of 1 hour at 37 degree Celsius while shaking at 200 rpm (C25 Incubator Shaker, New Brunswick Scientific). In the last step the cells were plated on LB-Agar plates containing 75 µg/mL antibiotic Carbenicillin (Gibco™, 10177012) and the plate was incubated at 37 degree Celsius overnight.

2.1.8 Bacterial overnight culture

For the overnight culture, 15 ml of Terrific Broth medium (Thermo Fisher Scientific, A1374301) and 75 µg/ml of the antibiotic Carbenicillin (Gibco™, 10177012) were added to a 50 ml falcon tube. Under sterile conditions, the prepared falcon tube was inoculated with a bacterial colony or a bacterial culture stored as a glycerol stock before incubation at 37 degree Celsius while shaking at 200 rpm (C25 Incubator Shaker, New Brunswick Scientific).

2.1.9 Plasmid extraction

For the isolation of plasmid DNA, an overnight culture was prepared the night before. The next day, the overnight culture was centrifuged at 11,000 g for 1 minute (Sorvall LYNX 4000 Superspeed Centrifuge, Thermo Scientific™), after which the cell pellet was used for the plasmid extraction. This method was performed according to the instructions of the NucleoSpin Plasmid Transfection-grade Kit (Macherey-Nagel, 740490.50) and the centrifugation steps were performed by using the Biofuge Pico centrifuge (Heraeus). Subsequently, the plasmid concentration was measured with the BioSpec-nano Microvolume Spectrophotometer (Shimadzu).

2.1.10 Glycerol stock preparation for long-term storage

The overnight bacterial culture was mixed with 20% sterile glycerol in a cryotube (Thermo Scientific™, 377267) and stored on ice for 10 minutes before transfer to –80 degree Celsius for long-term storage.

2.2 Cell culture methods

2.2.1 Passaging and plating of the BT cells

The medium for the patient-derived human glioblastoma cell lines BT12 and BT13 (Le Joncour et al., 2019) was prepared by adding 7.5 ml of 1M HEPES buffer (Lonza™ BioWhittaker™, BE17-737E), 10 ml B27 (Gibco™, 17504001), 5 ml of 200mM L-glutamine (Gibco™, 25030-024), and 5 ml of penicillin-streptomycin (Lonza™ BioWhittaker™, DE17-602E) to 500 ml of DMEM/F12 medium (Gibco™, 11320033). The cell plate was taken from 37 degree Celsius and all liquid containing the cells that grow as non-adherent spheroids was collected and transferred to a falcon tube. The falcon tube was centrifuged at 1200 rpm for 3 minutes at room temperature (Universal 32 R Centrifuge, Hettich) and the supernatant was carefully removed. Then 1 ml of the cell detachment solution Accutase (Sigma-Aldrich, A6964) was added to dissociate the cells from each other before the falcon tube was incubated at 37 degree Celsius for 3 minutes. Afterwards, 5 ml of medium was added, and the cells were resuspended before the falcon tube was centrifuged at 1200 rpm for 3 minutes at room temperature. The supernatant was removed, and 10 ml of fresh medium containing the growth factors EGF (Peprotech, AF-100-15) and FGF (Peprotech, AF-100-18B), was added to the falcon tube and resuspended. For plating of cells in suspension, a small amount of the cell-medium solution was transferred to a new Eppendorf tube and mixed with Trypan Blue Solution, 0.4% (Gibco™, 15250061) in a 1:1 ratio. Then the cells were counted by using a light microscope (Motic, AE31E), the BT cell solution was added to the 24-well plate and the plate was incubated at 37 degree Celsius.

For plating of coated BT cells, the Matrigel® Matrix Basement Membrane (Corning®, 356230) was taken from –20 degree Celsius and put on ice at 4 degree Celsius for approximately 3 hours. Then, DMEM/F12 medium (Gibco™, 11320033) was transferred to an Eppendorf tube and Matrigel was added in a 1:50 ratio and resuspended. The solution was transferred to a 24-well plate and the plate was incubated at 37 degree Celsius for one hour. Following the incubation period, the Matrigel-medium solution was removed from the 24-well plate. The BT cells were passaged, counted by using a light microscope and the BT cell solution was added to the 24-well plate, which was then incubated at 37 degree Celsius.

For maintenance, the cells were plated 1:2 on a new plate and 2 µl of the recombinant human epidermal growth factor (EGF, Peprotech, AF-100-15) and 1 µl of the

recombinant human fibroblast growth factor (FGF-basic, Peprotech, AF-100-18B) were added to the plate. The plate was then incubated at 37 degree Celsius. Every second day growth factors were added, and the cells were passaged once they reached a confluency level of 80 to 90 percent.

2.2.2 Passaging of HEK293FT cells

The medium for the human embryonic kidney cell line HEK293FT (Thermo Fisher Scientific) was prepared by adding 50 ml fetal bovine serum (Gibco™, 10500-064), 5 ml of 200mM L-glutamine (Gibco™, 25030-024) and 5 ml of penicillin-streptomycin (Lonza™ BioWhittaker™, DE17-602E) to 500 ml of DMEM (4.5 g/L D-glucose) medium (Gibco™, 11960044). The 10 cm cell dish was taken from 37 degree Celsius and the supernatant was carefully aspirated before the plate was washed twice with 5 ml of phosphate-buffered saline (PBS) (Medicago, 09-9400-100). Then 2 ml of the cell dissociation solution Trypsin EDTA (Lonza™ BioWhittaker™, BE17-161E) was added, and the cell dish was incubated at 37 degree Celsius for 3 to 5 minutes. In the next step all liquid was collected from the cell plate and transferred to a 15 ml falcon tube before centrifugation at 1200 rpm for 3 minutes at room temperature (Universal 32 R Centrifuge, Hettich). The supernatant was aspirated, and the cell pellet resuspended in 10 ml of DMEM medium. The cells were then plated 1:10 in a new cell culture plate and the plate was incubated at 37 degree Celsius. The cells were passaged once a confluency level of approximately 80% was reached.

2.2.3 Passaging of OP9 cells

The medium for the OP9 cells was prepared by adding 50 ml of fetal bovine serum (Gibco™, 10500-064), 5 ml of 200mM L-glutamine (Gibco™, 25030-024) and 5 ml of penicillin-streptomycin (Lonza™ BioWhittaker™, DE17-602E) to 500 ml of Minimum Essential (MEM) Alpha Medium (Gibco™, 22561-021). The 10 cm cell dish was taken from 37 degree Celsius and the supernatant was carefully aspirated before the plate was washed twice with 5 ml of PBS (Medicago, 09-9400-100). The PBS was removed, and 2 ml of the cell dissociation solution Trypsin EDTA (Lonza™ BioWhittaker™, BE17-161E) was added per 10 cm dish and then incubated for 3 to 5 minutes at 37 degree Celsius. After the incubation period, 8 ml of the medium was added to the cells and the cell solution was transferred to a falcon tube before centrifugation at 1200 rpm for 3 minutes (Universal 32 R Centrifuge, Hettich). The supernatant was removed, and 10 ml of fresh medium was added. The cells were then plated 1:2 in a new cell culture

plate and the plate was incubated at 37 degree Celsius. The cells were passaged once a confluency level of 80% was reached.

2.2.4 Thawing of cells

The cryotubes (Thermo Scientific™, 377267) containing cells were taken from –80 degree Celsius and put on ice. After the cells were thawed, 1 ml of appropriate medium was added to the cryotubes and resuspended. In the next step, a new falcon tube with 9 ml of medium was prepared, before the cell solution was transferred from the cryotube to the falcon tube and resuspended in the medium. Subsequently the falcon tube was centrifuged at 1200 rpm for 3 minutes at room temperature (Universal 32 R Centrifuge, Hettich) before the supernatant was removed. As a last step, 10 ml of new medium were added to the falcon tube and the solution was transferred to a new plate. The plate containing the cells was then incubated at 37 degree Celsius.

2.2.5 Freezing of cells

The cell plate containing cells in suspension was taken from 37 degree Celsius and all cells were collected in a falcon tube and centrifuged at 1200 rpm for 3 minutes (Universal 32 R Centrifuge, Hettich) at room temperature before the supernatant was removed. Then an Eppendorf tube containing 90% of medium and 10% of the cryopreservation agent Dimethyl sulfoxide (DMSO, Sigma-Aldrich, D8418) was prepared and 1 ml of this solution was added to the falcon tube containing the cells and subsequently resuspended. Next all the solution in the falcon tube was transferred to a cryotube (Thermo Scientific™, 377267), which was labelled and kept on ice for 10 minutes before being transferred to the –80 degree Celsius for long-term storage. To freeze adherent cells, the cell dishes were treated with Trypsin EDTA (Lonza™ BioWhittaker™, BE17-161E) first to dissociate the cells from the plate and then centrifuged at 1200 rpm for 3 minutes (Universal 32 R Centrifuge, Hettich) before freezing the cells according to the instructions for suspension cells.

2.2.6 MTT assay

The volume of cell-medium solution in the plate wells was estimated and the required amount of the MTT reagent Thiazolyl Blue Tetrazolium Bromide (Sigma Aldrich, M5655) was added to each well (10% of the volume/well). After incubating the plate for 2 hours at 37 degree Celsius, MTT lysis buffer (50 ml of 20% SDS, 83 µl of 37% HCl and 100 µl of ddH_2O) was added to the wells. The plate was then incubated overnight at 37 degree Celsius overnight. The next day, the plate was measured at an

optical density of 540 nm by using a Microplate reader (FLUOstar® Omega, BMG Labtech). The data from the Microplate reader was analyzed using the Omega Data Analysis software (BMG Labtech).

2.3 Exosome methods

2.3.1 Sample collection for exosome isolation

The HEK293FT or OP9 cells were cultured on 15x15 cm cell culture dishes. Two days before the sample collection, the medium was changed to medium containing exosome depleted fetal bovine serum (Gibco™, A2720801). After 48 hours of incubation time at 37 degree Celsius, the supernatant from the cell culture dishes was transferred to a 50 ml falcon tube. The falcon tubes containing the supernatants were centrifuged at 300 g for 5 minutes (Universal 32 R Centrifuge, Hettich) at room temperature before transferring the supernatant to a new 50 ml falcon tube and centrifuging at 1250 g for 10 minutes (Universal 32 R Centrifuge, Hettich) at room temperature. In the last step, the supernatant was transferred to another 50 ml falcon tube and then filtered with a sterile Minisart Syringe filter (Pore Size 0.22 µm, Sartorius, 16532) into a 50 ml falcon tube before storage at -20 degree Celsius until further use.

2.3.2 Exosome isolation

The exosomes were isolated from HEK293FT or OP9 derived supernatant that had been prepared and stored at -20 degree Celsius as described in the previous chapter. The supernatants were then thawed in a warm water bath and concentrated by using the Millipore Amicon ultra-15 100 K (Merck, UFC910024) filter tubes. The upper chamber of the filter tubes was filled with the supernatant and then the tubes were centrifuged at 4000g for 10 minutes (Sorvall LYNX 4000 Superspeed Centrifuge, Thermo ScientificTM) at room temperature. In the next step, the flow-through was discarded before the upper chamber was refilled with the supernatant and another centrifuge step took place. This process was repeated until all supernatant had been concentrated to about 500 µl. While the centrifuge process took place, the qEVoriginal Size Exclusion Column (Izon Science, SP1) was taken from 4 degree Celsius and equilibrated at room temperature before washes with 20 ml of PBS (Medicago, 09-9400-100). Here the time was measured for collecting 5 ml in a measuring cylinder, to ensure the quality of the qEVoriginal Size Exclusion Column. Once the washes were done, the 500 µl of supernatant were added slowly and evenly on top of the Size Exclusion column (SEC) membrane. After all the supernatant had passed through the

filter, PBS was added again, and the fractioning took place. The first 3 ml were discarded, while the last 1.5 ml, which contained the exosomes were collected in an Eppendorf tube and stored at –80 degree Celsius. Then the Size Exclusion column was washed with 40 ml of PBS and the time was measured for collecting 5 ml in a measuring cylinder. This was followed by 7 washes with a 20% EtOH-PBS solution. Then, 2 ml of the 20% EtOH-PBS solution was left in the column, the bottom and top were sealed with parafilm (Sigma-Aldrich, P7543) and the SEC column was stored at 4 degree Celsius.

2.3.3 Transfection of plasmids with/without exosomes

Exosome aliquots, each containing about 50 µl of isolated exosomes, were taken from –80 degree Celsius and thawed at 37 degree Celsius. Approximately five to six hours later, the transfection reagent Fugene HD (Promega, E2311) was equilibrated at room temperature for 10 minutes, while the plasmids were taken from –20 degree Celsius and thawed at room temperature. For each transfection, tube A with 150 µl PBS (Medicago, 09-9400-100) and tube B with 75 µl of PBS were prepared. Then 3 µg of plasmid was added to tube A, while 12 µl of Fugene HD solution was added to tube B. Subsequently the tubes A and B were incubated for 5 minutes at room temperature before mixing them. After another incubation period of 15 minutes at room temperature, the exosomes were added to the plasmid/Fugene HD solution. The transfected exosomes were then incubated at 37 degree Celsius for about 18 hours.

To test the functionality of constructed plasmids, tube A with 150 µl PBS and 3 µg of the new plasmid construct was prepared, while tube B contained 75 µl PBS and 12 µl of the transfection reagent Fugene HD. Before mixing, both tube A and B were incubated at room temperature for 5 minutes. Then, another incubation period of 15 minutes at room temperature took place before the solution was inoculated directly into the target cells. The cells were incubated at 37 degree Celsius for up to 4 days before imaging. During imaging, the cells were checked for apoptotic signs in case of targeting plasmids or fluorescent signal if non-targeting plasmids containing fluorescent proteins were used.

2.3.4 Inoculation of target cells with transfected exosomes

After incubation of the transfected exosomes at 37 degree Celsius, they were transferred to 10 K Amicon® Ultra 0.5 mL Centrifugal Filters (Merck, UFC501024). The filter tubes were topped up to 450 µl with PBS (Medicago, 09-9400-100) before

centrifugation at 6100 g for 15 minutes (Minispin Plus G, Eppendorf) at room temperature. The flow-through was discarded and PBS was added to the filter tubes once more before the tubes were centrifuged at 6100 g for 20 minutes (Minispin Plus G, Eppendorf) at room temperature. Subsequently, this step was repeated at 6100 g for 25 minutes (Minispin Plus G, Eppendorf) at room temperature. Next, the filter tubes were inverted into a new 10 K Amicon® Ultra 0.5 mL Centrifugal Filter tubes and centrifuged at 1000 g for 2 minutes (Minispin Plus G, Eppendorf) at room temperature before the filters were discarded and the transfected exosomes, remaining in the tubes, inoculated into the target cells.

In some experiments we used a variation of the protocol with addition of DNAse I treatment. After incubation of transfected exosomes at 37 degree Celsius, they were transferred to Amicon® Ultra 0.5 mL Centrifugal Filters with the filter pore size varying from 50K (Merck, UFC505024) to 100K (Merck, UFC510024). Then the filter tubes were topped up to 450 µl with PBS (Medicago, 09-9400-100) before centrifugation at 4000 g for 10 minutes (Minispin Plus G, Eppendorf) at room temperature. Subsequently the flow-through was discarded and DNAse I (New England Biolabs, M0303S) was mixed with PBS in a ratio of 1:80 and added to the filter tubes. The filter tubes were filled with 400 µl of this mixture and incubated for 15 minutes at room temperature. After the incubation, the filter tubes were centrifuged at 4000 g for another 10 minutes (Minispin Plus G, Eppendorf) before the flow through was discarded and the tubes were topped up to 450 µl with PBS and centrifuged again at 4000 g for 10 minutes (Minispin Plus G, Eppendorf) at room temperature. The filter was then inverted in a new Amicon® Ultra 0.5 mL Centrifugal Filter tube and centrifuged at 1000 g for 2 minutes (Minispin Plus G, Eppendorf) at room temperature. As the last step, the filter was discarded, and the transfected exosomes were inoculated into the target cells.

2.4 Virus-related methods

2.4.1 Production of lentiviruses

All experiments including lentiviruses were performed in the Biosafety Level 2 (BSL-2) laboratories due to the moderate risk associated with the viruses upon swallowing or contact with the skin.

One day prior the transfection, the HEK293FT cells were passaged and cultured for 24 hours to a confluence of approximately 80%. On the next day, the DMEM (4.5 g/L D-glucose) medium (Gibco™, 11960044) and Opti-MEM (Gibco™, 31985-047) were

warmed up at 37 degree Celsius and the transfection reagent Fugene HD (Promega, E2311) was kept at room temperature for 10 minutes before the use. The cell culture medium was carefully changed to 5 ml of fresh and warm DMEM medium and the transfection mixes were prepared in tubes A and B. In tube A, the target plasmid, the packaging plasmid CMV delta 8.3 (Addgene) and the envelope plasmid CMV-G (Addgene) were mixed with Opti-MEM, while tube B contained the transfection reagent Fugene HD in Opti-MEM. The tubes were flicked to mix and incubated at room temperature for 5 minutes before combination to one tube AB. Then this tube was flicked to mix again and incubated for another 20 minutes at room temperature. The transfection mix in tube AB was then added dropwise to the HEK293FT cells before the cells were transferred to the 37 degree Celsius incubator in the BSL-2 room. After an incubation period of 48 hours, the medium containing the lentivirus was collected and filtered through a sterile Minisart 0.45 µm syringe filter (Sartorius, 16537) into a 50 ml falcon tube and the cells were supplemented with 5 ml of fresh and warm DMEM medium. The falcon tube was sealed with Parafilm (Sigma-Aldrich, P7543) and stored at 4 degree Celsius overnight. After 24 hours of incubation time, this process was repeated and the virus containing medium was collected and sterile filtered, before combination with the medium collected a day prior. The harvested viral particles were aliquoted in 1 ml aliquots in cryotubes (Thermo Scientific™, 377267) and stored at -80 degree Celsius.

2.4.2 Transduction of OP9 cells with virus supernatant

The OP9 cells were transduced with the lentivirus to enhance the targeting of the glioblastoma cells. For this, the murine, mesenchymal progenitor OP9 cell line was cultured and passaged. After a centrifugation step at 1200 rpm for 3 minutes (Universal 32 R Centrifuge, Hettich), 10 ml of MEM Alpha Medium (Gibco™, 22561-021) was added and the cells were plated into 3 wells of a 6-well plate and 6 wells of a 24-well plate. The plates were incubated at 37 degree Celsius for 24 hours. Then, 0.5 µl, 1 µl, 2 µl, 3 µl or 4 µl of the antibiotic Puromycin (Sigma-Aldrich, P8833) was added to the 24-well plate but leaving one well untreated as a negative control. The plate was then incubated in the BSL-2 room at 37 degree Celsius and carefully monitored. After 2 to 3 days, the appropriate Puromycin antibiotic (Sigma-Aldrich, P8833) concentration was decided, needed for an antibiotic growth curve selection. The next day the virus transduction was performed by adding 2 ml of OP9 cells and 2 ml of virus containing medium to a 6-well plate before adding the cationic polymer Hexadimethrine bromide

(Polybrene, Sigma-Aldrich, 107689) in a 1:1,000 concentration and incubating at 37 degree Celsius in the BSL-2 room for 2 days. Polybrene was added during this step, since it has been shown to enhance the transduction efficiency of cells with lentivirus (H. E. Davis et al., 2002). After 24 hours the medium was changed and after 48 hours the cells were divided to two 10 cm cell culture dishes. One of the 10 cm plates was supplemented with 30 µl of the antibiotic Puromycin, while the second plate was supplemented with 40 µl of Puromycin. Since the lentiviral vector resulted in resistance to the antibiotic Puromycin, this antibiotic was added to select the transduced cells while eliminating the non-transduced cells (Addgene 2019). The virus transduced OP9 cells were passaged once they reached a confluent state and were continuously cultured on 15x15 cm plates, at which point the cells could be used for the isolation of exosomes ("2.3.1 Sample collection for exosome isolation").

2.5 Imaging

2.5.1 Thermo EVOS FL

The target cells with the transfected exosomes were imaged using the Widefield Microscope Thermo EVOS FL provided by the Biomedicum Imaging Unit (https://www2.helsinki.fi/en/infrastructures/bioimaging/biomedicum-imaging-unit). The cells containing exosomes transfected with targeting plasmids were monitored for signs of apoptosis, such as cell shrinkage and blebbing, while the cells inoculated with exosomes transfected with control non-targeting plasmids but including mCherry (red) or enhanced green fluorescent protein (eGFP) fluorophores were checked for a corresponding signal.

2.5.2 Bioluminescence Imaging

To test for bioluminescence in the OP9 cells in order to confirm the presence of the NanoLuc® (NLuc) luciferase enzyme in the cells and thereby a successful virus transduction, a plate containing control conditions with the wildtype OP9 cells and test conditions, which included virus transduced OP9 cells, was imaged. The Nano-Glo® Luciferase Assay System (Promega, N1110) was added to the control and test conditions and subsequently this plate was imagined using the Spectral Instruments Imaging Lago small animal imaging system, which was provided by the Biomedicum Imaging Unit – IVI. A successful virus transduction of the OP9 cells was confirmed in case of bioluminescent signal due to the expression of the NLuc luciferase enzyme in the cells, which was only present in virus transfected cells.

3 Results

3.1 Targeting plasmids for common genetic alterations in glioblastoma cells lack transfection efficiency

This research project focuses on plasmids targeting genetic alterations in glioblastoma cells. The selected plasmids encode oncoproteins or tumor suppressor proteins, which have been shown to decrease proliferation in cancer cells when silenced or upregulated. The first step in setting up the exosome-based gene therapy comprised of testing the most efficient plasmids with the potential to decrease proliferation of glioblastoma cells.

The targeting plasmids and their corresponding controls (non-targeting plasmids) are displayed in Table 5. Targeting plasmids were constructed to silence the expression of the oncogene EGFRvIII and to overexpress the tumor suppressor genes PTEN and TP53.

Table 5. Targeting plasmids and the corresponding control non-targeting plasmids.

Targeting plasmids	Non-targeting plasmids
pNL1.1_CMV_mCherry_shEGFRvIII	pNL1.1_CMV_mCherry_shNEGATIVE
pNL1.1_pmir21_PTEN	pNL1.1_pmir21_gfp_shNEGATIVE
pNL1.1_pmir21_TP53	pNL1.1_CMV_mCherry_shNEGATIVE

These plasmids had been constructed prior to this research project and therefore I started by transfecting them into exosomes and inoculating them into the glioblastoma cells to test their efficacy. The addition of exosomes was repeated two more times to increase the efficiency of the targeting. The cells were imaged 48 hours after the 3rd inoculation with transfected exosomes using the Widefield Microscope Thermo EVOS FL. The BT12 glioblastoma cells, treated with a combination of the targeting plasmids, showed signs of cell death, while the cancer cells treated with the non-targeting plasmids proved the successful uptake of the transfected exosomes by the target cells as judged by the fluorescent signals for mCherry and eGFP. The majority of the control cells did not show any signs of apoptosis (Figure 1).

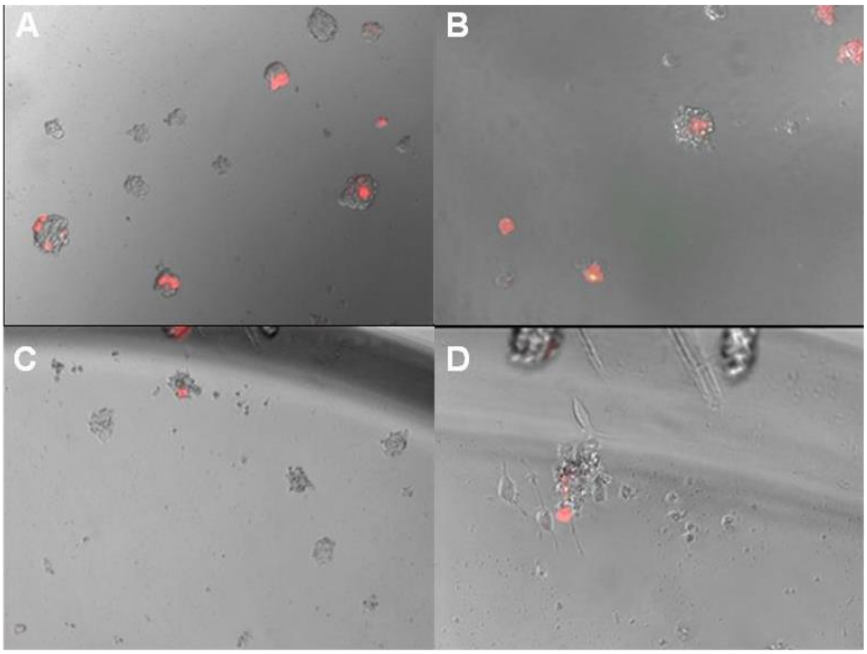


Figure 1. (A-B) Representative Thermo EVOS FL micrographs of BT12 cells treated with the non-targeting control plasmids pNL1.1_CMV_mCherry_shNEGATIVE, pNL1.1_pmir21_gfp_shNEGATIVE and pNL1.1_CMV_mCherry_shNEGATIVE encoding mCherry and eGFP, show corresponding red and green fluorescence signal. Magnification 4x. (C-D) Representative Thermo EVOS FL micrographs of BT12 cells treated with the targeting plasmids pNL1.1_CMV_mCherry_shEGFRvIII, pNL1.1_pmir21_PTEN and pNL1.1_pmir21_TP53, encoding EGFRvIII, PTEN or TP53, show signs for apoptosis. Red signal represents the targeting plasmid pNL1.1_CMV_mCherry_shEGFRvIII encoding mCherry. Magnification 4x (C), 10x (D).

Three inoculations of exosomes were necessary to detect visible fluorescence signal (Figure 1). The size of these plasmids was approximately 5700 bp and the large size probably decreased the transfection efficiency and the uptake of the plasmids into the exosomes. Therefore, we decided to test smaller plasmids, which utilize apoptosis-inducing proteins to increase the efficiency and decrease the number of necessary inoculations.

3.2 Design of targeting plasmids utilizing apoptosis-inducing proteins to increase transfection efficiency in glioblastoma cells

The following plasmids were designed and subsequently tested.

The targeting plasmid encoding Granzyme B enzyme (GzmB) was constructed by purchasing the hpmir21-GzmB vectors from the VectorBuilder. Subsequently, GzmB was subcloned (2.1.5 Ligation) into the Miniplasmid pUCmu to reduce the size of the plasmid construct, which positively influences the efficiency of the exosome transfection (Hardee et al., 2017). The successful insertion of GzmB into pUCmu was

verified using colony PCR. The gel electrophoresis picture is displayed in Appendix A. Due to the anticipated low transfection efficiency of plasmid DNA into the exosomes, the gene of interest needed to be very efficiently expressed in the target cells. To achieve this, we added a nuclear localization sequence (NLS) and a DNA translocation sequence (DTS) to the plasmid. The DTS sequence (GGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCA) resulted in a plasmid translocation from the cytosol to the nucleus, thereby enhancing gene transcription, while the NLS sequence (CCAAAAAAGAAGAGAAAGGTA) enhanced the translocation of the GzmB protein to the nucleus, where the damage took place. The corresponding control for the GzmB targeting plasmid was created by substituting the GzmB sequence with the fluorescent protein eGFP by using the assembly cloning HiFi (2.1.5 Ligation). The primer sequences used for the cloning and verification of the targeting and non-targeting plasmids are displayed in Appendix B. The maps displaying the targeting plasmid GzmB and the corresponding control including eGFP are shown in Figures 2 and 3.

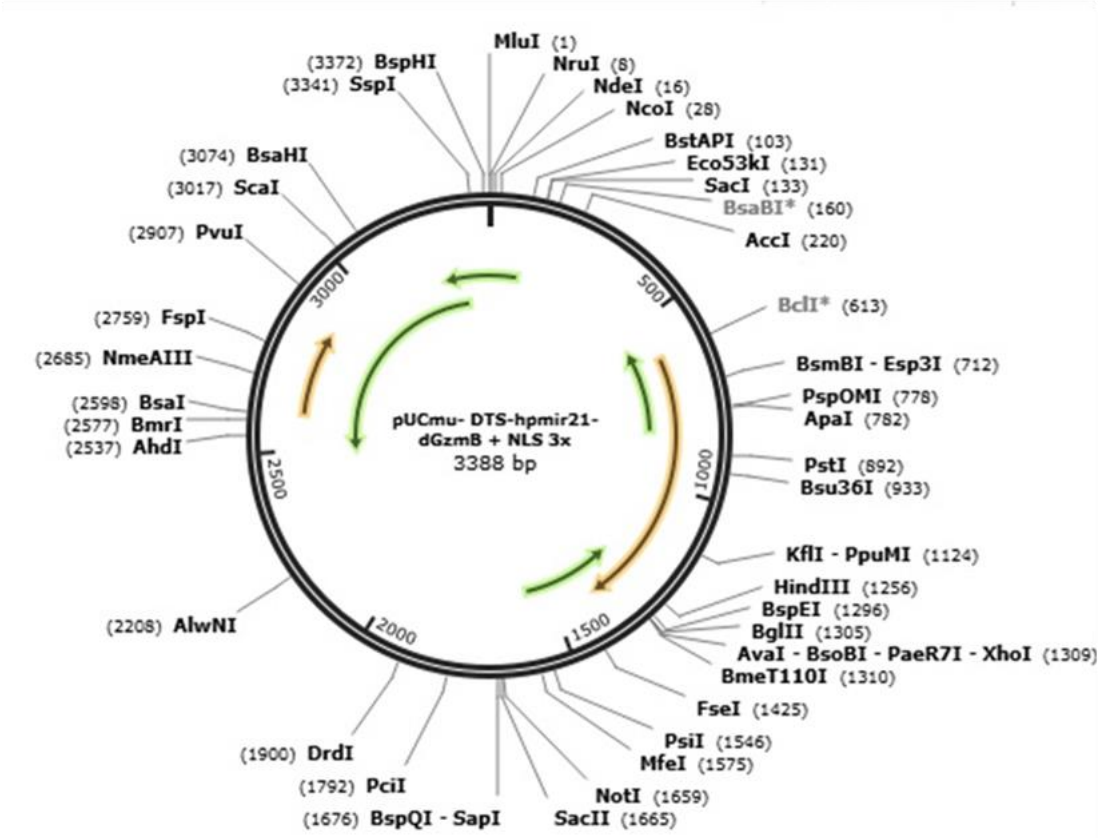


Figure 2. Map of the pUCmu-DTS-hpmir21-dGzmB + NLS 3x by Snapgene.

Figure 3. Map of the pUCmu-DTS-hpmir21-eGFP by SnapGene.

The targeting plasmid encoding the sequence for Diphtheria toxin fragment A (DTA) was constructed by purchasing the hpIL13RA2-DTA vectors from VectorBuilder and subsequently subcloning the DTA into the Miniplasmid pUCmu by using the DNA assembly HiFi from NEB (2.1.5 Ligation) to decrease the size of the plasmid construct. The successful cloning was verified by colony PCR. The gel electrophoresis picture is displayed in Appendix A. The DTA plasmid was further modified by adding the DTS sequence resulting in the plasmid translocation from the cytosol to the nucleus to improve the gene transcription process. The NLS sequence was not added to the plasmid since the DTA effect occurs in the cytoplasm by inhibition of the protein synthesis. The DTA sequence of the plasmid was then substituted with the sequence encoding the fluorescent protein mCherry (2.1.5 Ligation) to create the corresponding control for the DTA plasmid. The primer sequences used for the cloning and verification of the targeting and non-targeting plasmids are shown in Appendix B. The maps

displaying the targeting plasmid with DTA and the corresponding mCherry control are shown in Figures 4 and 5.

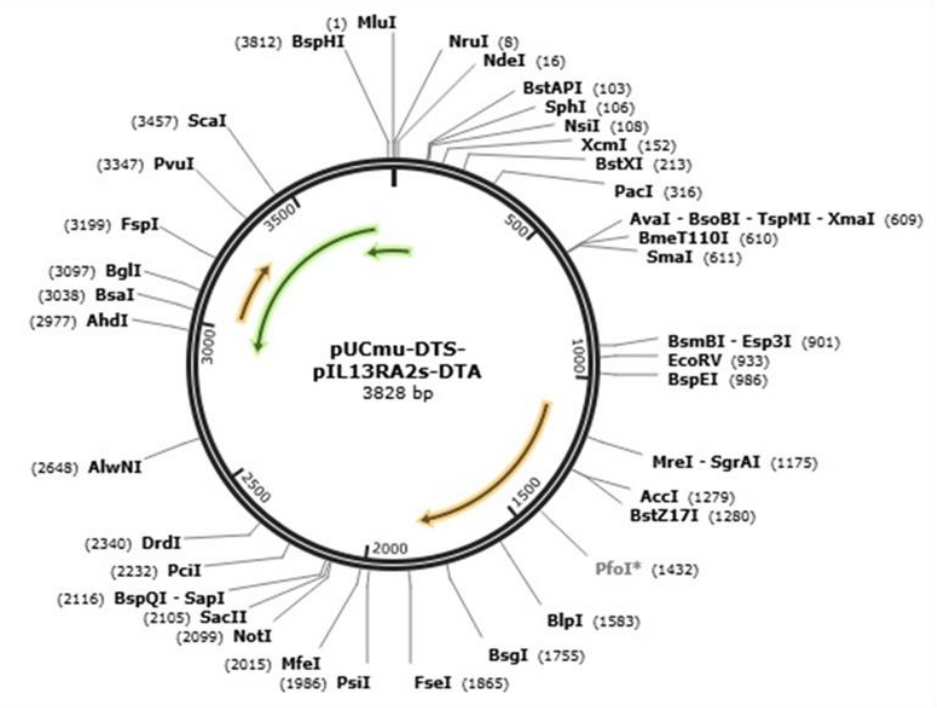


Figure 4. Map of the pUCmu-DTS-plL13RA2s-DTA by SnapGene.

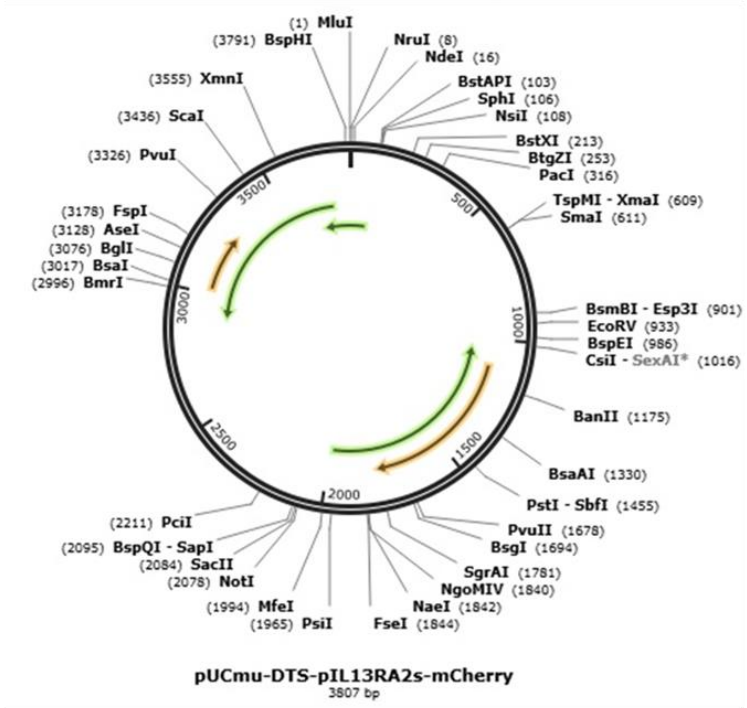


Figure 5. Map of the pUCmu-DTS-plL13RA2s-mCherry by SnapGene.

The final targeting plasmid constructs, and their corresponding controls are displayed in Table 6.

Table 6. Targeting plasmids and corresponding control plasmids for Granzyme B and Diphtheria toxin fragment A.

Targeting plasmid	Control
pUCmu-DTS-hpmir21-dGzmB + NLS 3x	pUCmu-DTS-hpmir21-eGFP
pUCmu-DTS-pIL13RA2s-DTA	pUCmu-DTS-pIL13RA2s-mCherry

3.3 Isolation of exosomes as carriers for gene therapy in glioblastoma cells

3.3.1 Exosomes derived from the HEK293FT cell line used as main exosome source

The HEK293FT laboratory stock was selected for the isolation of exosomes due to the fast growth and simple culture conditions. In addition, due to yet unknown reasons, HEK293FT cells target glioblastoma cells very efficiently.

To confirm that the effect on the target cells is exclusively due to the plasmids that have been transfected into the exosomes and not due to external plasmid, we isolated HEK293FT-derived exosomes, transfected the exosomes with the non-targeting plasmid pNL1.1_CMV_mCherry_shNEGATIVE including the mCherry protein, and inoculated the transfected exosomes into a different set of HEK293FT cells. The control conditions did not contain exosomes, but only the plasmid and the transfection reagent Fugene HD. Both, the control and targeted cells were treated with the DNAse I enzyme to remove extra plasmids that had not been taken up by the exosomes during the transfection process. The cells were imaged 48, 96 and 144 hours after the inoculation using the Widefield Microscope Thermo EVOS FL (Figure 6).

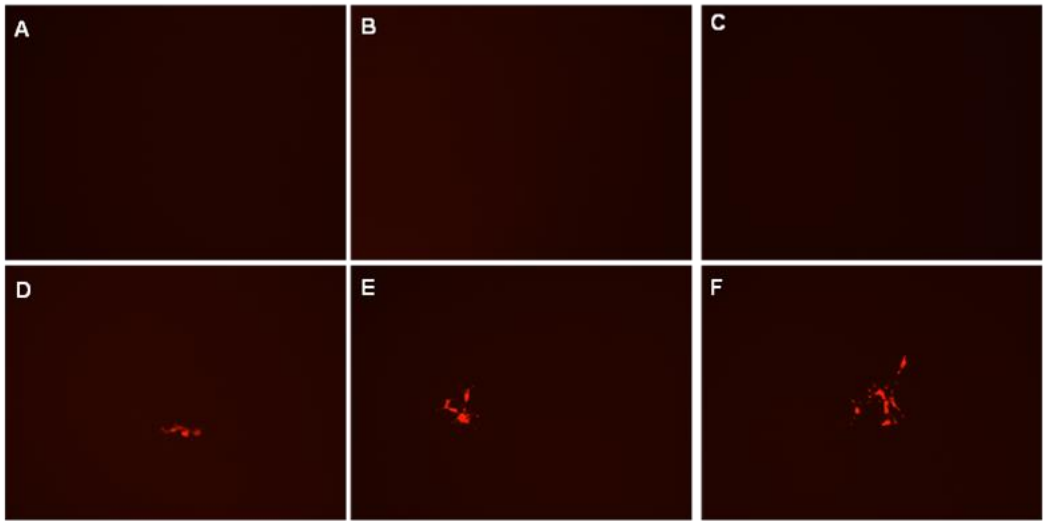


Figure 6. Representative Thermo EVOS FL micrographs of cells treated with the control plasmid pNL1.1_CMV_mCherry_shNEGATIVE without exosomes 2 (A), 4 (B) or 6 (C) days after inoculation. Representative Thermo EVOS FL micrographs of cells treated with plasmid pNL1.1_CMV_mCherry_shNEGATIVE and exosomes 2 (D), 4 (E) or 6 (F) days after inoculation. Magnification 4x.

The results confirmed that addition of the DNAse I enzyme eliminated any external plasmids not taken up by the exosomes. Thus, only the plasmids taken up by exosomes showed a positive fluorescent signal.

3.3.2 Exosomes derived from the virus-transduced OP9 cell line used as alternative exosome source

We used the murine mesenchymal progenitor OP9 cell line as an alternative source for the isolation of exosomes. For that purpose, OP9 cells were transduced with lentiviruses, encoding the tumor homing peptide iRGD and NanoLuc® luciferase enzyme. The successful lentiviral transduction was confirmed using the Bioluminescence imaging after addition of the Nano-Glo® Luciferase Assay System (Figure 7). As a result, the exosomes isolated from the virus transfected OP9 cells, carried the homing peptide iRGD on the surface, enabling a more efficient uptake of the exosomes by glioblastoma cells.

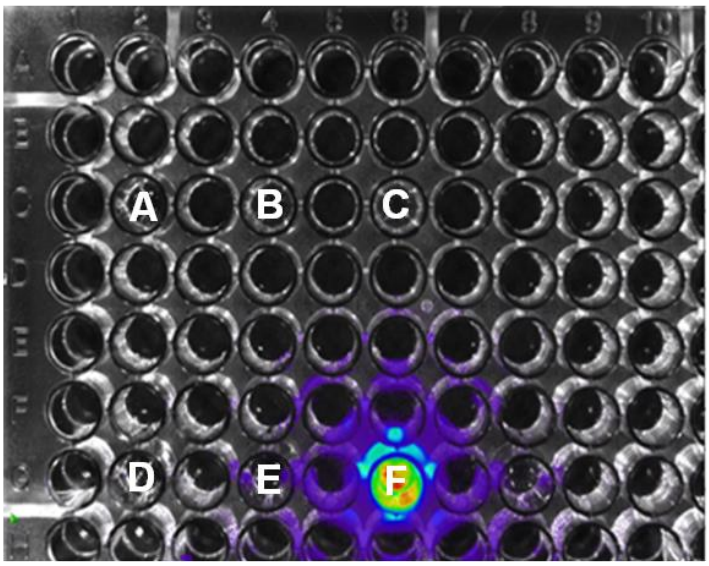


Figure 7. Bioluminescence Imaging. (A-C) OP9 cells, without Nano-Glo® Luciferase Assay System. (D-E) Lentivirus transduced OP9 cells, producing NLuc exosomes without Nano-Glo® Luciferase Assay System. (F) Lentivirus transduced OP9 cells, producing NLuc exosomes with Nano-Glo® Luciferase Assay System.

In addition to the use of the iRGD homing peptide, another goal was to track the exosomes *in vivo* via the expression of the NanoLuc® luciferase enzyme by virus-transduced cells. The expression of luciferase enzyme by the exosomes was verified by treatment of the cells with Furimazine, a substrate to the NanoLuc. Treatment resulted in high-intensity luminescence only when the lentivirus transduced cells were treated with Nano-Glo® Luciferase Assay System (Figure 8).

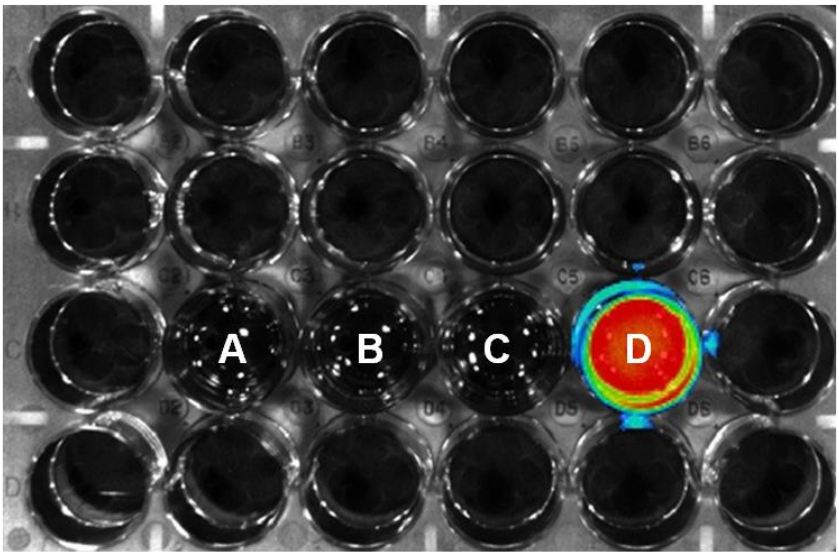


Figure 8. Bioluminescence Imaging. (A) OP9 cell culture media alone. (B) OP9 cell culture media with Furimazine. (C) WT OP9 cells with Furimazine. (D) NLuc exosomes isolated from virus transfected OP cells with Furimazine.

3.4 Study of targeting and non-targeting plasmids in glioblastoma cells for exosome-based gene therapy

3.4.1 Testing of plasmid construct efficiency for exosome-based gene therapy in glioblastoma cells

The efficiency in inducing apoptosis of the targeting plasmids encoding the Granzyme B and Diphtheria toxin fragment A and their corresponding controls encoding eGFP and mCherry (Table 6) was tested by transfecting the BT12 cells with plasmids without exosomes. Cells were imaged using the Widefield Microscope Thermo EVOS FL 72 hours after transfection. Our results showed a fluorescence signal in cells transfected with the control plasmids and signs of cell death of cells transfected with the targeting plasmids (Figure 9).

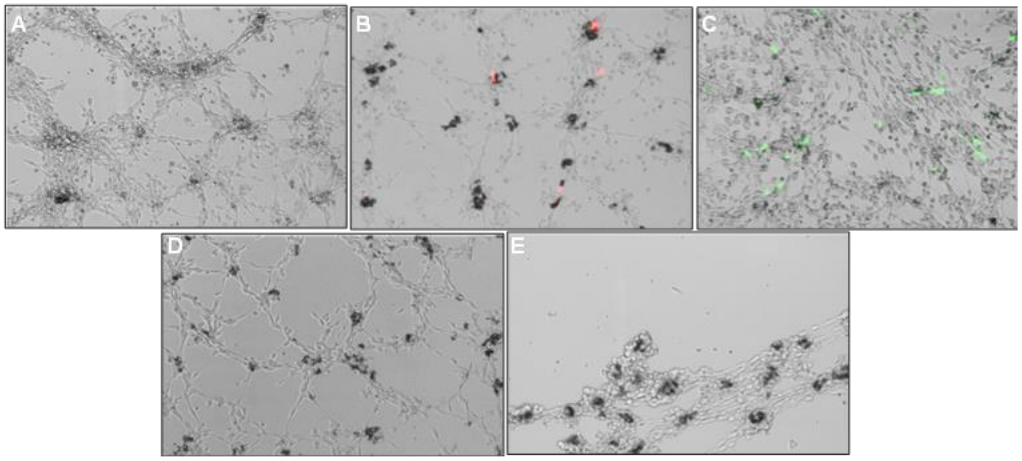


Figure 9. (A) Representative Thermo EVOS FL micrographs of BT12 cells without treatment. (B-E) Representative Thermo EVOS FL micrographs treated with the non-targeting plasmid pUCmu-DTS-pIL13RA2s-mCherry (B), the non-targeting plasmid pUCmu-DTS-hpmir21-eGFP (C), the targeting plasmid pUCmu-DTS-pIL13RA2s-DTA (D), and the targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x (E). Magnification 10x.

To further test the efficacy of the plasmid constructs, an MTT assay was conducted to measure the effect of the different plasmids on cell viability. MTT assay confirmed the decrease in cell viability for the targeting plasmids pUCmu-DTS-pIL13RA2s-DTA and pUCmu-DTS-hpmir21-dGzmB + NLS 3x (Figure 10). Cells transfected with the non-targeting plasmid pUCmu-DTS-hpmir21-eGFP showed an increased cell viability compared to the control non-transfected cells, while the non-targeting plasmid pUCmu-DTS-pIL13RA2s-mCherry resulted in a minor decrease of the cell viability (Figure 10).

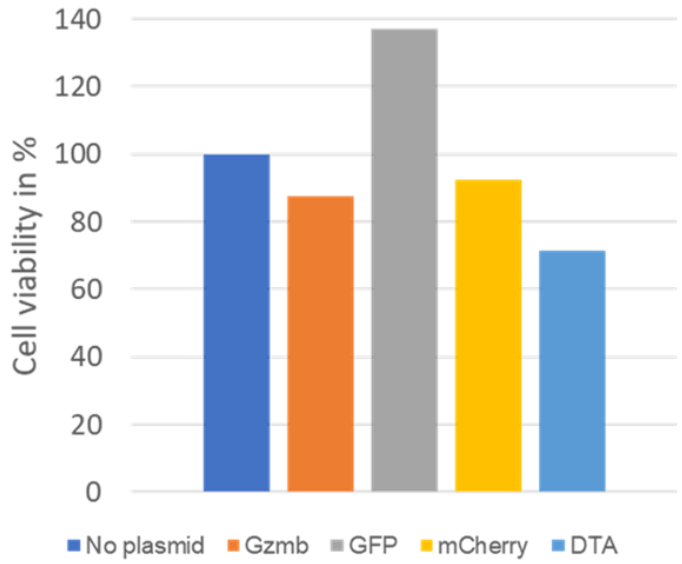


Figure 10. MTT assay in BT12 cells for plasmid testing. No exosome addition. One inoculation with 3 µg of plasmid.

3.4.2 Enhancing of targeting plasmid encoding Granzyme B shows improved transfection efficiency in glioblastoma cells

The cell death induced by Granzyme B is one of the mechanisms used by the human body to kill tumor cells and therefore, this enzyme is a potential candidate for inducing apoptosis in glioblastoma cells. First, I transfected the isolated exosomes with the targeting plasmid hpmir21-dGzmB following inoculation of exosomes to BT12 glioblastoma cells. Treated cells were imaged 72 hours after the 2nd inoculation with transfected exosomes using the Widefield Microscope Thermo EVOS FL. The Granzyme B expressing cells showed signs of apoptosis in comparison with the control-treated cells (Figure 11).

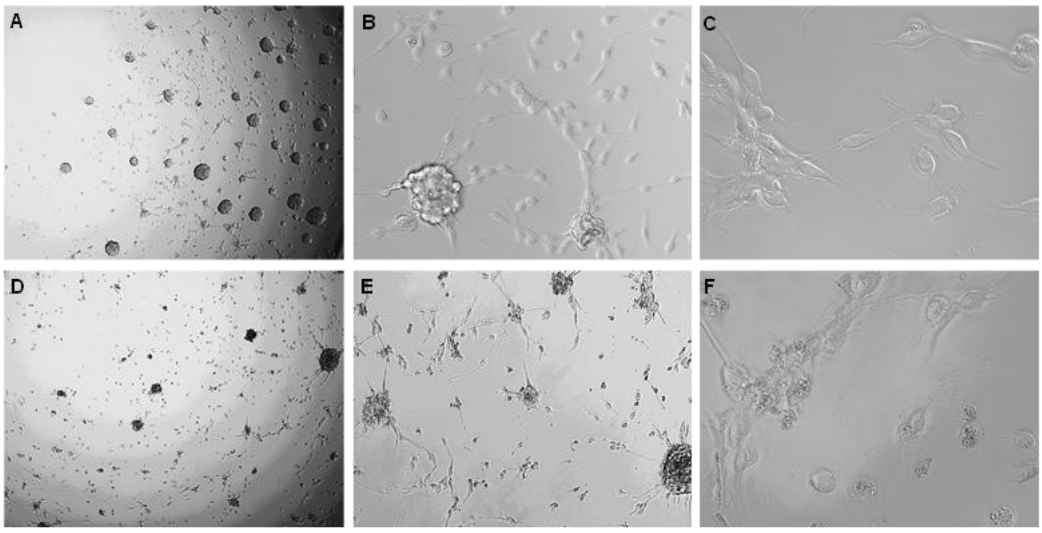


Figure 11. (A-C) Representative Thermo EVOS FL micrographs of BT12 cells without treatment. Magnification 4x (A), 10x (B), 20x (C). (D-F) Representative Thermo EVOS FL micrographs of BT12 cells treated with targeting plasmid hpmir21-dGzmB. Magnification 4x (D), 10x (E) and 20x (F).

Therefore, we decided to further increase its targeting efficiency by addition of the DTS and NLS sequences in the plasmid (DTS-hpmir21-dGzmB + NLS 3x).

The BT12 glioblastoma cells were treated with the isolated enhanced Granzyme B plasmid transfected exosomes and imaged 96 hours after the 1st exosome inoculation using the Widefield Microscope Thermo EVOS FL. MTT assay to assess the cell viability was performed on day 5 after the 1st exosome inoculation.

Both, the imaging (Figure 12) and the MTT assay (Figure 13) verified the cell death and decreased cell number of the cells treated with granzyme B transfected exosomes compared to the control cells treated with the non-targeting plasmid.

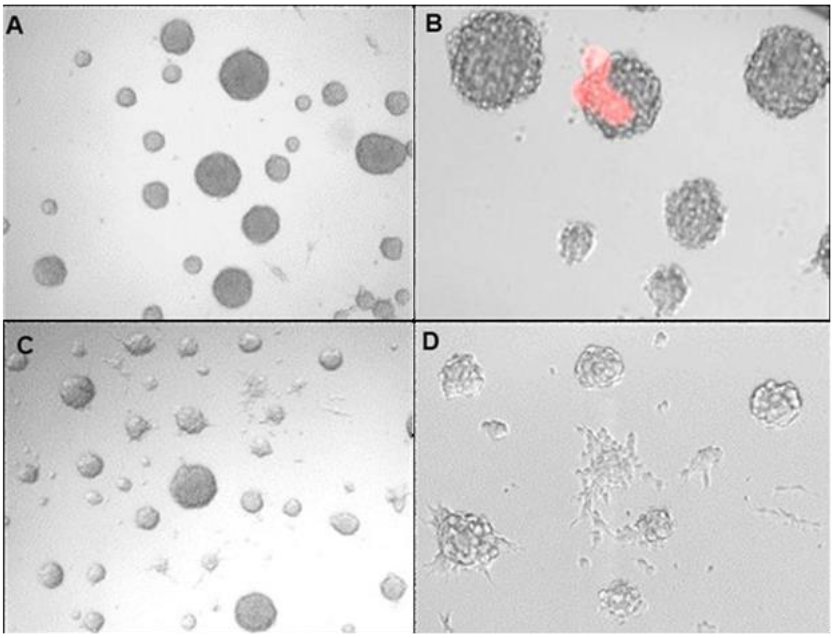


Figure 12. (A-B) Representative Thermo EVOS FL micrographs of BT12 cells treated with the non-targeting plasmid pNL1.1_CMV_mCherry_shNEGATIVE. Magnification 4x (A), 10x (B). (C-D) Representative Thermo EVOS FL micrographs of BT12 cells treated with the targeting plasmid DTS-hpmir21-dGzmB + NLS 3x. Magnification 4x (C), 10x (D).

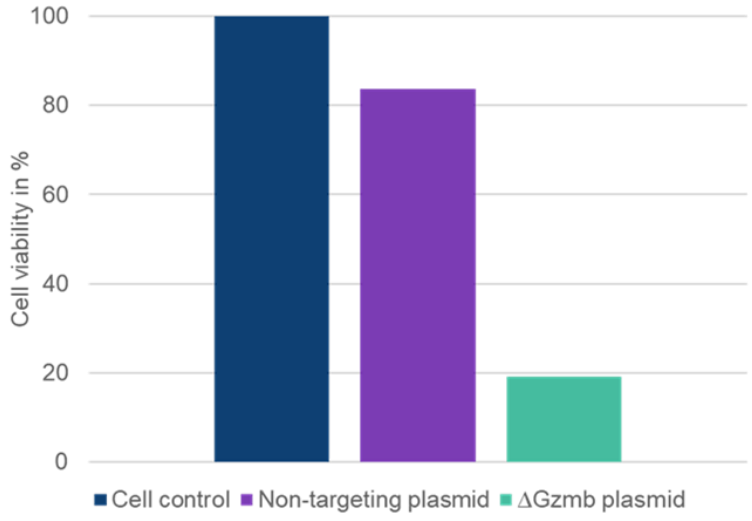


Figure 13. MTT assay in BT12 cells for exosome-based therapy. HEK293 exosomes. One inoculation with enhanced Granzyme B plasmid.

Granzyme B reduced the cell viability by 81% compared to the control non-treated cells (Figure 13) thereby confirming the efficacy of this approach. Since the plasmid size influences the transfection efficiency, we decided to study if decreasing the size of the Granzyme B encoding plasmid would further increase the efficiency of the targeting.

3.4.3 Targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x results in highest transfection efficiency

The enhanced Granzyme B encoding plasmid containing the DTS and NLS sequences was subcloned into the miniplasmid pUCmu, which decreased the size of the overall construct by 600 bp and thereby potentially influenced the transfection efficiency. I then transfected the pUCmu plasmid construct into the HEK293-derived exosomes and treated the BT12 glioblastoma cells. 72 hours after the 1st exosome inoculation the cells were imaged using the Widefield Microscope Thermo EVOS FL and an MTT assay was performed the following day. The images showed apoptosis and a decreased cell number of the cells treated with the granzyme B exosomes in comparison to the control cells treated with the non-targeting plasmid (Figure 14).

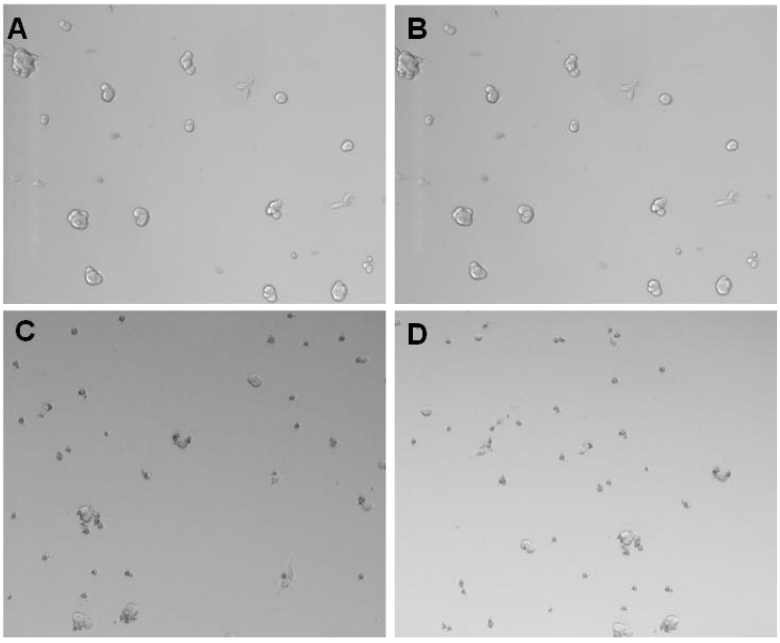


Figure 14. (A-B) Representative Thermo EVOS FL micrographs of BT12 cells treated with non-targeting plasmid pNL1.1_CMV_mCherry_shNEGATIVE. Magnification 10x. (C-D) Representative Thermo EVOS FL micrographs of BT12 cells treated with targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x. Magnification 10x.

Viability of the cells treated with the granzyme B exosomes was reduced by 93,1% compared to the non-treated control cells (Figure 15). Reduction in cell viability was

about 12% more with the smaller plasmid confirming the effect of the size reduction in the transfection efficiency. However, also viability of the control cells treated with the non-targeting plasmid pNL1.1_CMV_mCherry_shNEGATIVE reduced by 32,8% compared to the non-treated control cells (Figure 15). Overall, the results with the plasmid construct pUCmu-DTS-hpmir21-dGzmB + NLS 3x were promising.

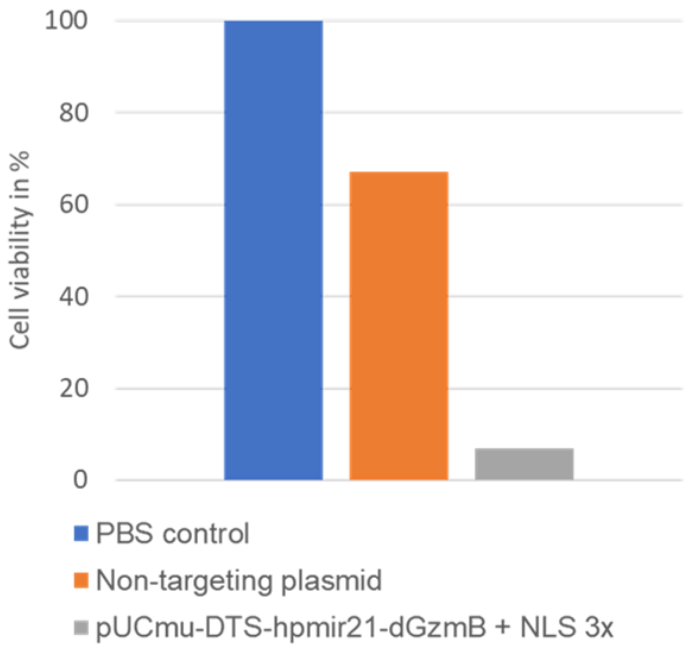


Figure 15. MTT assay in BT12 cells for exosome-based therapy. HEK293 exosomes. One inoculation with pUCmu-DTS-hpmir21-dGzmB + NLS 3x.

To make sure that only the plasmids, that have been taken up by the exosomes affected the target cells, I added the DNAse I enzyme during the centrifugation of the filter tubes with the transfected exosomes, followed by the inoculation into the BT12 glioblastoma cells. This enzyme cleaves DNA and therefore degraded all plasmids outside the exosomes, ensuring that the effect on target cells was a result of the exosome-based gene therapy. Cell viability was determined by using the MTT assay 96 hours after the inoculation of the transfected exosomes.

Cell viability decreased by 15,9% in the cells treated with the targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x compared to the control cells treated with the non-targeting plasmid pNL1.1_CMV_mCherry_shNEGATIVE (Figure 16). However, the decrease in cell viability after the DNAse I addition was less compared to the cell viability without the addition of DNAse I (Figure 15), indicating that part of this detected effect was due to external plasmids not taken up by the exosomes.

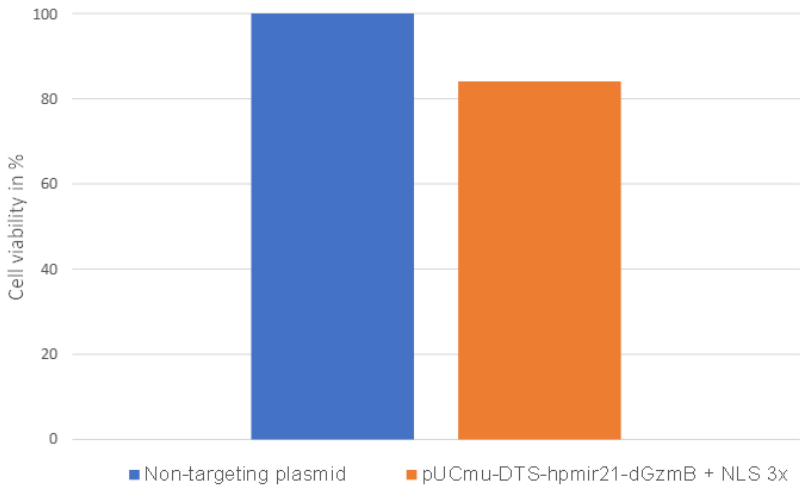


Figure 16. MTT assay in BT12 cells for exosome-based therapy. HEK293 exosomes. One inoculation with 3 µg of pUCmu-DTS-hpmir21-dGzmB + NLS 3x.

3.4.4 Study of varying conditions to test potential effect of reaction components on glioblastoma cell viability

To clarify the effect of the exosomes, the transfection reagent, and the plasmids not taken up by the exosomes on the target cell viability, different conditions were analyzed.

In every set-up, five different conditions were tested with the first condition set as a control containing only cells, while the second condition contained cells incubated with the PBS buffer. For the third condition, a mock exosome transfection was carried out without addition of exosomes, to make sure that only the plasmids taken up by the exosomes led to an effect on target cells. The fourth condition comprised an exosome transfection, in which the plasmid was substituted with PBS, to test the effect of the transfection reagent Fugene HD. For the fifth condition an exosome transfection was conducted, and all components were added. For all five conditions, the DNAse I enzyme was added during the centrifugation steps preceding the inoculation into the BT12 glioblastoma cells to remove external plasmids outside of the exosomes.

In addition, the parameters tested included varying pore sizes of the centrifugal filter tubes (50 kDa or 100 kDa) used during the centrifugation of the filter tubes with the transfected exosomes before inoculation into the BT12 glioblastoma cells, the source

of the exosomes (HEK293FT cells or virus transfected OP9 cells) and the BT12 cells grown either in suspension or as an adherent culture.

The cells were imaged 96 hours after the 1st exosome inoculation using the Widefield Microscope Thermo EVOS FL and an MTT assay was performed the following day. Both cell imaging (Figure 17) as well as the MTT cell viability assay data (Figure 18) indicate that the pUCmu-DTS-hpmir21-dGzmB + NLS 3x plasmid transfected into HEK293-derived exosomes and isolated using the 50 kDa centrifugal filter tubes resulted in decreased cell viability of approximately 11% compared to control condition 4 containing Fugene HD and exosomes, which showed the lowest cell viability out of the other conditions (Figure 18). For the condition 3 where no exosomes were present, an increase in cell viability was detected, indicating that the addition of DNAse I enzyme successfully removed extra plasmids, not taken up by the exosomes. Thus, no effect on the viability of target cells was detected (Figure 18). Overall, the highest decrease in cell viability (approximately 22%) resulted when cells were treated with the exosomes carrying the targeting plasmids.

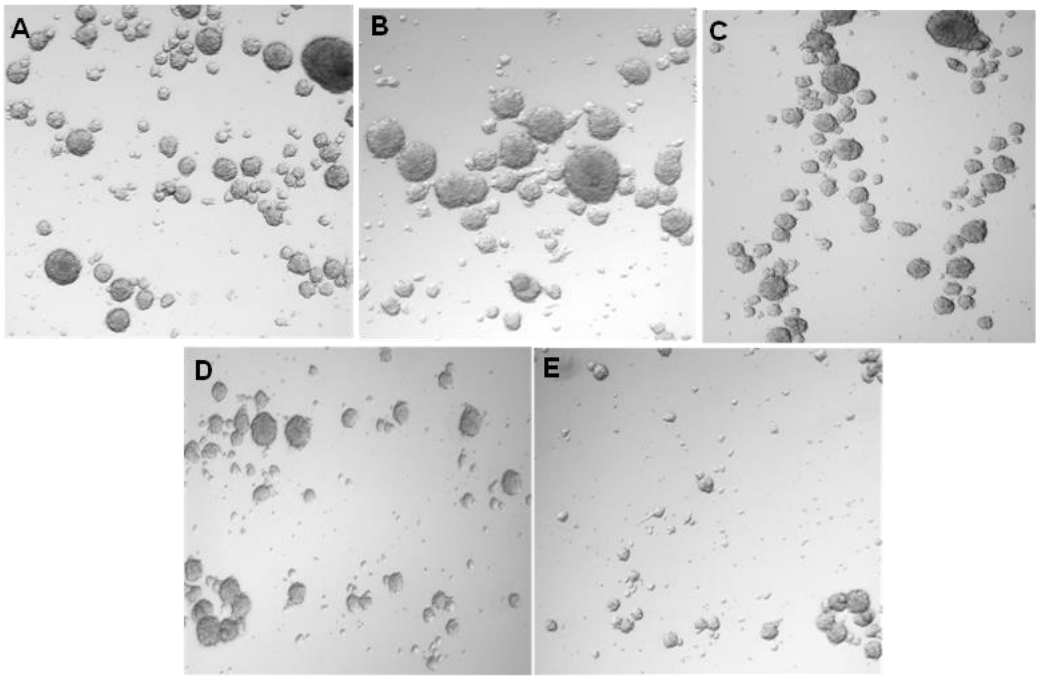


Figure 17. (A) Representative Thermo EVOS FL micrographs of non-treated BT12 cells. (B-E) Representative Thermo EVOS FL micrographs of BT12 cells treated with PBS buffer (B), targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x without exosomes (C), exosomes without targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x (D) and targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x and exosomes (E). Magnification 4x.

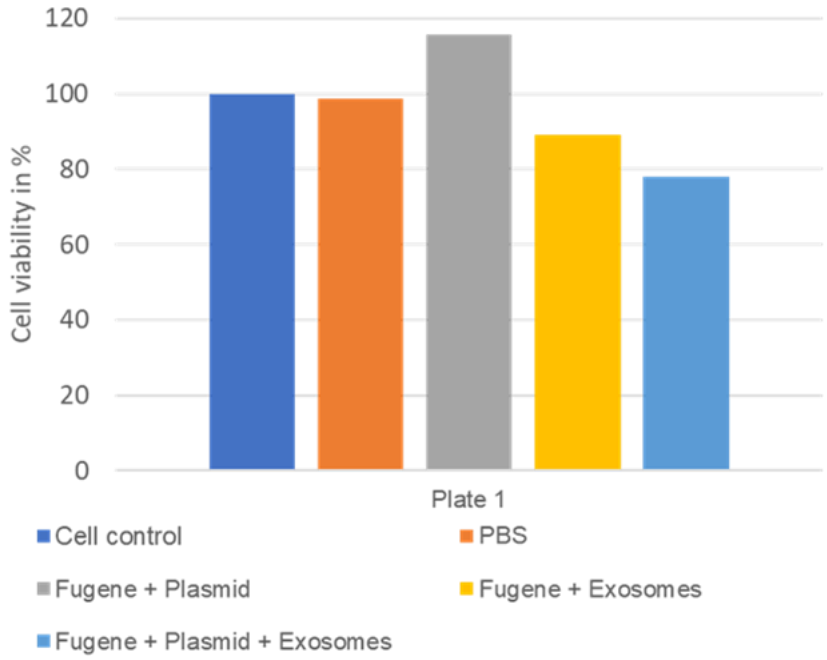


Figure 18. MTT assay for BT12 cells in suspension for exosome-based therapy. HEK293 exosomes. Use of 50 kDa centrifugal filter tubes. One inoculation with 3 µg of pUCmu-DTS-hpmir21-dGzmB + NLS 3x.

The same five conditions were applied to exosomes isolated from the virus transfected OP9 cells and isolated using the 100 kDa centrifugal filter tubes.

The cells were imaged 96 hours after the 1st exosome inoculation using the Widefield Microscope Thermo EVOS FL and an MTT assay was performed the following day. Both cell imaging (Figure 19) as well as the MTT cell viability results (Figure 20) showed a decrease in cell viability in the conditions 3 to 5 which all contained Fugene HD (Figure 20). The highest decrease in cell viability (about 58%) was detected in the exosome therapy (condition 5) (Figure 20). Cell viability in this group was about 15% lower compared to the conditions 3 and 4 (Figure 20). This is in accordance with the results obtained with the HEK293FT-derived exosomes.

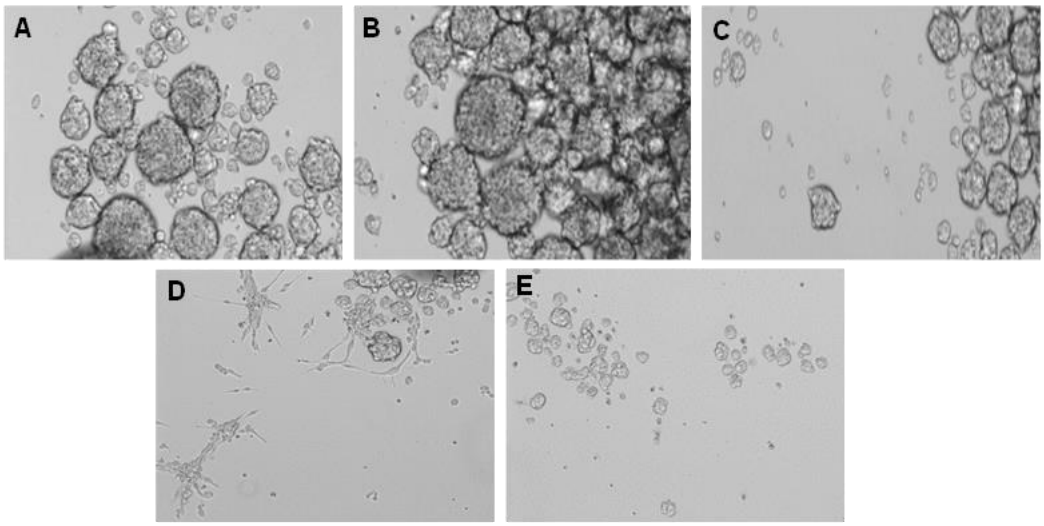


Figure 19. (A) Representative Thermo EVOS FL micrographs of non-treated BT12 cells. (B-E) Representative Thermo EVOS FL micrographs of BT12 cells treated with PBS buffer (B), targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x without exosomes (C), exosomes without targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x (D), and targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x and exosomes (E). Magnification 10x.

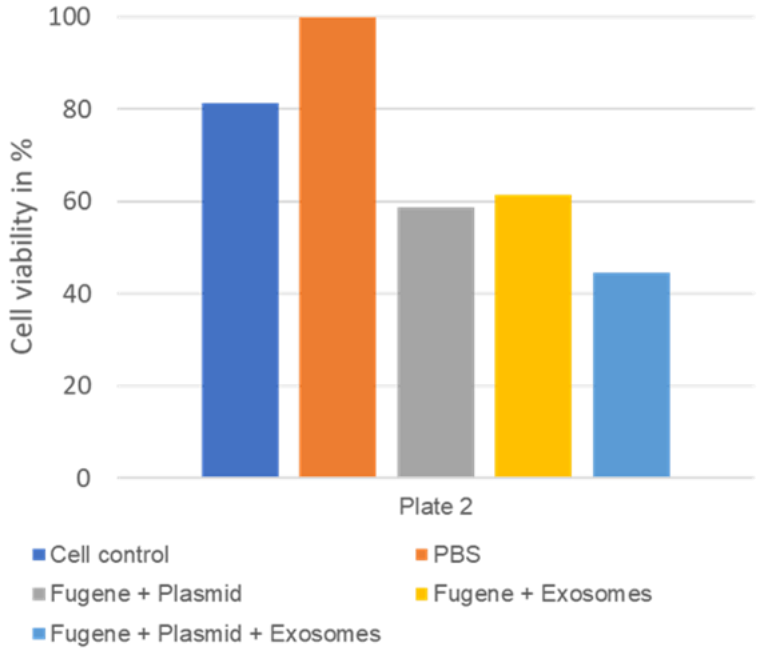


Figure 20. MTT assay in BT12 cells in suspension for exosome-based therapy. Virus transfected OP9 exosomes. Use of 100 kDa centrifugal filter tubes. One inoculation with 3 µg of pUCmu-DTS-hpmir21-dGzmB + NLS 3x.

Then, the same five conditions were applied to the exosomes isolated from HEK293FT cells and isolated by using 100 kDa centrifugal filter tubes. The cells were imaged 96

hours after the 1st exosome inoculation using the Widefield Microscope Thermo EVOS FL and an MTT assay was performed the following day. Cell imaging (Figure 21) showed less cells in conditions 3 and 4 compared to the non-treated control cells, which was confirmed by the MTT cell viability results (Figure 22). The MTT assay showed that the cell viability of conditions 3 and 4 was decreased by approximately 13%, while the viability of cells treated with the exosome-based gene therapy showed the highest decrease (approximately 19%) of cell viability compared to conditions 3 and 4 (Figure 22). The result of highest cell viability decrease in cells treated with the exosome-based gene therapy is in accordance with the results obtained for the exosomes isolated from the HEK293FT cells and isolated by using 50 kDa filters. The decrease in the viability of PBS treated cells differs from all previous experiments that have not shown any decrease in cell viability. Therefore, it represents an outlier and can be discarded.

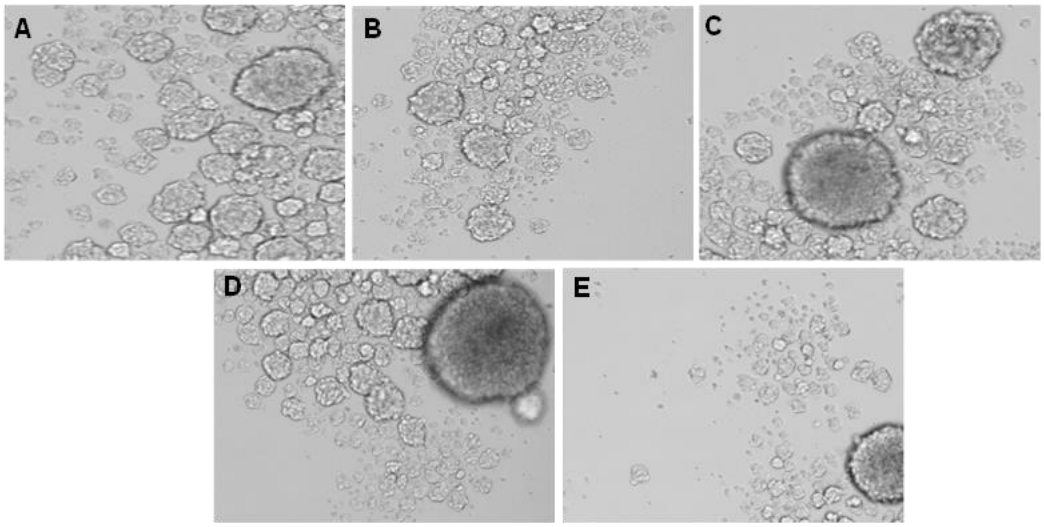


Figure 21. (A) Representative Thermo EVOS FL micrographs of non-treated BT12 cells. (B-E) Representative Thermo EVOS FL micrographs of BT12 cells treated with PBS buffer (B), targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x without exosomes (C), exosomes without targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x (D), and targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x and exosomes (E). Magnification 10x.

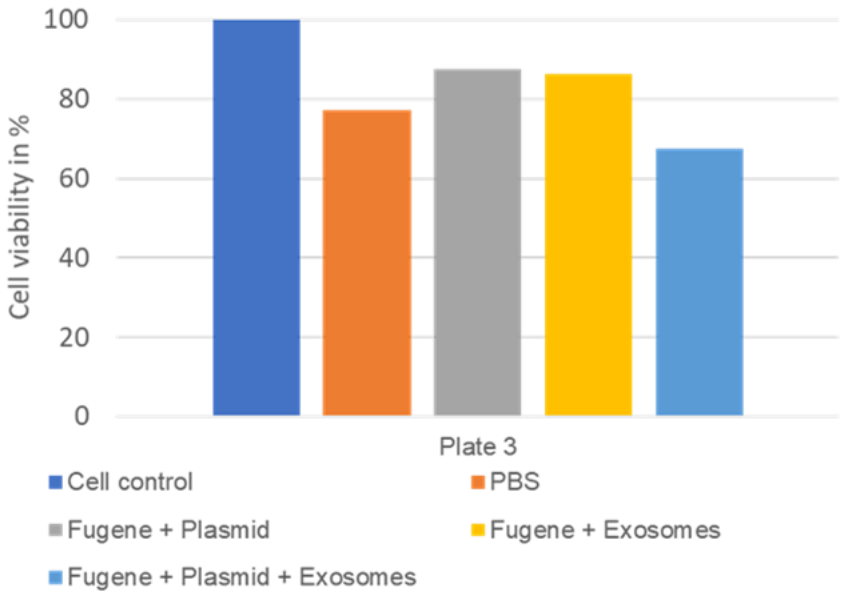


Figure 22. MTT assay in BT12 cells in suspension for exosome-based therapy. HEK293 exosomes. Use of 100 kDa centrifugal filter tubes. One inoculation with 3 µg of pUCmu-DTS-hpmir21-dGzmB + NLS 3x.

Taken together, the decrease in viability of the cells treated with exosome gene therapy has varied in between 11% to 19% compared to the control conditions, indicating that the effect on the target cells is not dependent on the source of the exosomes or the pore sizes of the centrifugal filter tubes. Furthermore, the effect of the exosome therapy on cell viability overrides that of the transfection reagent Fugene HD or the effect of the exosomes, indicating specific effect of the exosome-based gene therapy.

Next, the BT12 cells were plated as adherent cells by using Matrigel. In addition to this change, a new condition was added, where the transfection reagent Fugene HD was replaced with PBS buffer to test if any passive diffusion of plasmids into the exosomes without the presence of a transfection reagent occurred. The cells were imaged 96 hours after the 1st exosome inoculation using the Widefield Microscope Thermo EVOS FL and an MTT assay was performed the following day. Cell imaging (Figure 23) showed less cells in the exosome-based gene therapy treated cells. This was confirmed by the MTT cell viability assay (Figure 24). The highest decrease (approximately 14%) in viability occurred in cells treated with exosome gene therapy compared to the other conditions 3 to 5 (Figure 24).

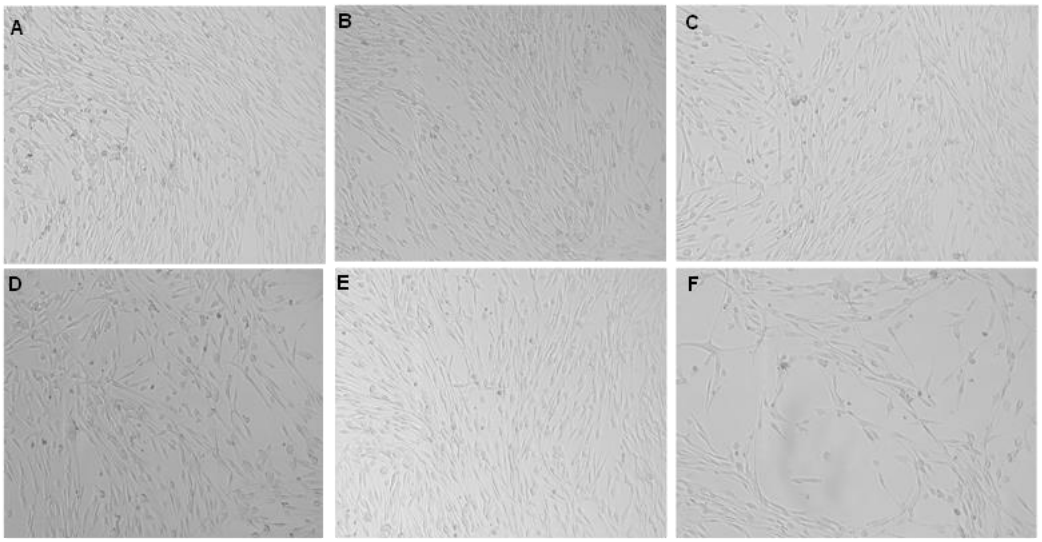


Figure 23. (A) Representative Thermo EVOS FL micrographs of non-treated BT12 cells. (B-F) Representative Thermo EVOS FL micrographs of BT12 cells treated with PBS buffer (B), targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x without exosomes (C), exosomes without targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x (D), targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x and exosomes without transfection reagent Fugene HD (E) and targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x and exosomes (F). Magnification 10x.

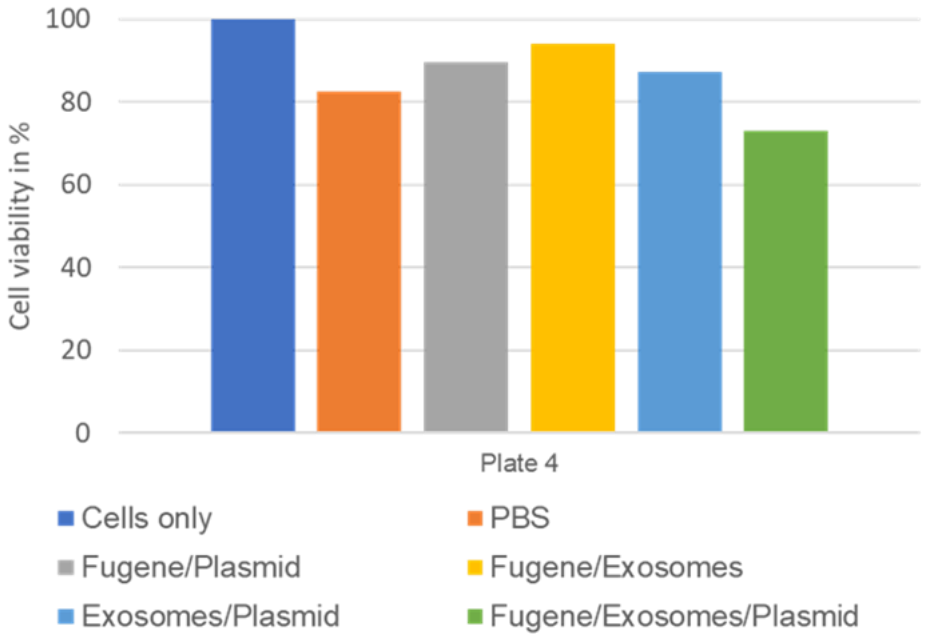


Figure 24. MTT assay in BT12 cells in adherent condition for exosome-based therapy. HEK293 exosomes. Use of 50 kDa centrifugal filter tubes. One inoculation with 3 µg of pUCmu-DTS-hpmir21-dGzmB + NLS 3x.

After studying the different conditions and set-ups, the viability of cells treated with exosome therapy containing the pUCmu-DTS-hpmir21-dGzmB + NLS 3x plasmid consistently showed the lowest number of live cells varying between 10 – 20% decrease compared to the other control conditions.

3.4.5 Targeting plasmid encoding Diphtheria toxin fragment A reduces cell viability in glioblastoma cells

The Diphtheria toxin fragment A (DTA) is characterized by a high toxicity due to an inhibition of protein synthesis by inactivating the eukaryotic elongation factor 2 (EF2). EF2 is highly expressed in a variety of malignant tumor cells and its expression shows correlation with the cancer progression and recurrence, making it a potential candidate for an anti-cancer agent. To study the efficacy of DTA in exosome-based gene therapy plasmid DTS- pIL13RA2s-DTA was transfected into the HEK293FT-derived exosomes and inoculated to the BT12 glioblastoma cells. This experiment was repeated twice with two independent exosome inoculations. The cells were imaged 72 hours after the 2nd inoculation of transfected exosomes using the Widefield Microscope Thermo EVOS FL and an MTT assay was performed the following day. Cell imaging (Figure 25) showed less cells and signs of apoptosis when cells were treated with the targeting plasmid encoding DTA. This was confirmed by the MTT cell viability results (Figure 26).

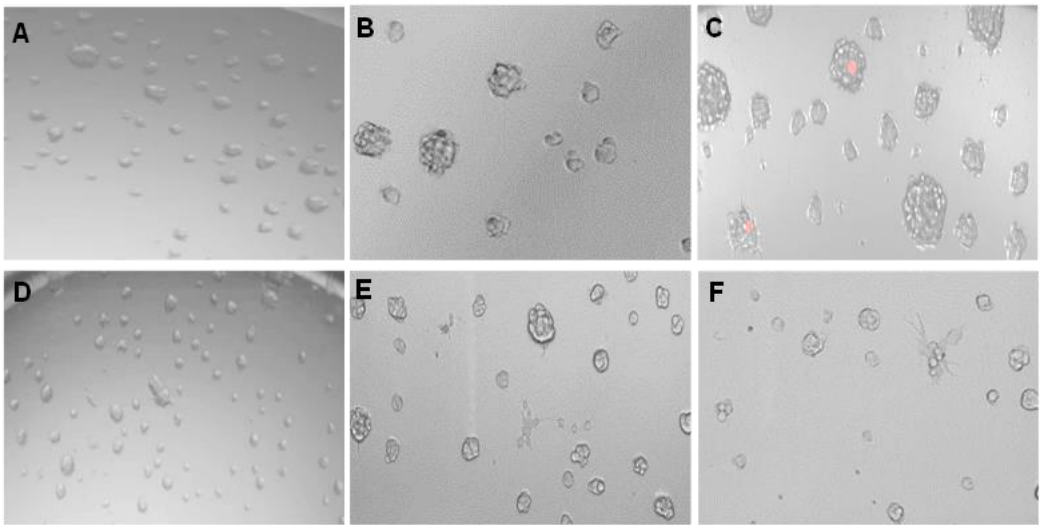


Figure 25. (A-C) Representative Thermo EVOS FL micrographs of BT12 cells treated with non-targeting plasmid pNL1.1_CMV_mCherry_shNEGATIVE encoding mCherry, show corresponding red fluorescence signal. Magnification 4x. (A), 10x (B-C). (D-F) Representative Thermo EVOS FL micrographs of BT12 cells treated with targeting plasmid DTS-pIL13RA2s-DTA encoding DTA. Magnification 4x (D), 10x (E-F).

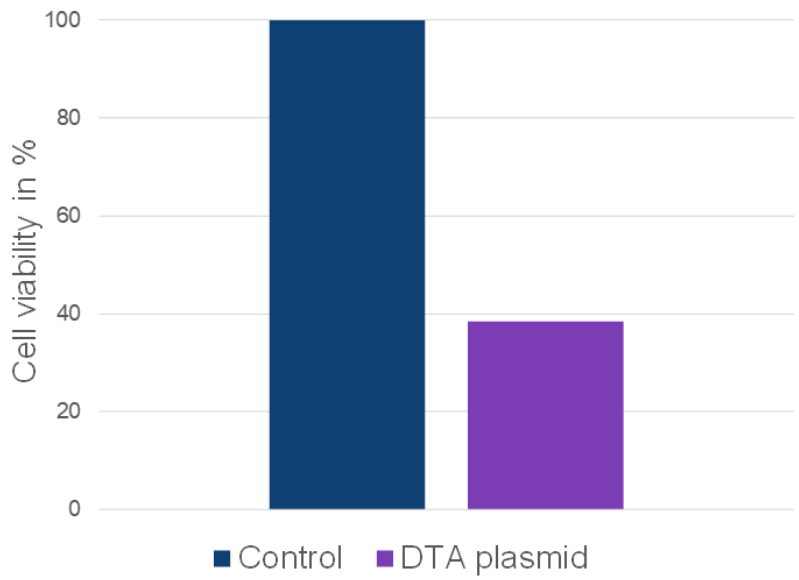


Figure 26. Average of MTT assays in BT12 cells in suspension for exosome-based therapy including DTA plasmid. HEK293 exosomes. Use of 10 kDa centrifugal filter tubes. Two inoculations with 3 µg of DTS-pIL13RA2s-DTA.

An additional independent exosome inoculation was carried out, for which the cells were imaged 96 hours after the first inoculation of the transfected exosomes using the Widefield Microscope Thermo EVOS FL and an MTT assay was performed the following day. Cell imaging (Figure 27) showed less cells and signs of apoptosis when cells were treated with the targeting plasmid encoding DTA. This was confirmed by the MTT cell viability results (Figure 28), indicating that one inoculation was sufficient to observe a toxic effect in the treated cells.

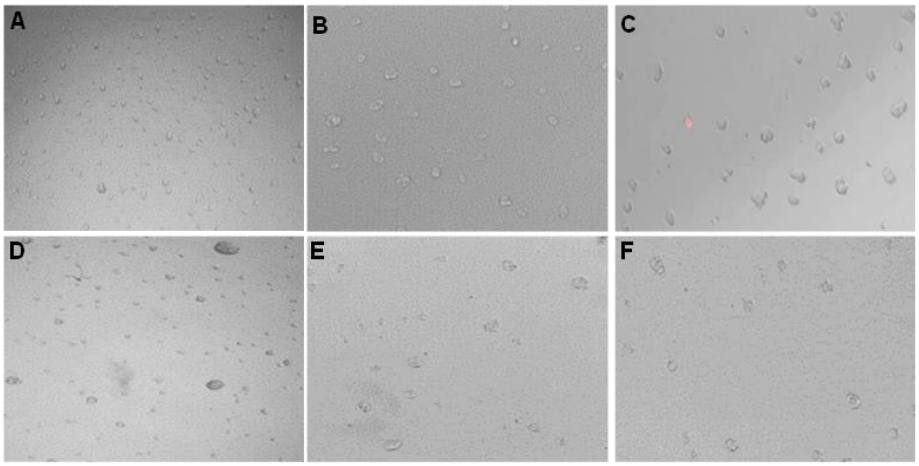


Figure 27. (A-C) Representative Thermo EVOS FL micrographs of BT12 cells treated with non-targeting plasmid pNL1.1_CMV_mCherry_shNEGATIVE encoding mCherry, show corresponding red fluorescence signal. Magnification 4x. (A), 10x (B-C). (D-F) Representative Thermo EVOS FL micrographs of BT12 cells treated with targeting plasmid DTS-pIL13RA2s-DTA encoding DTA. Magnification 4x (D), 10x (E-F).

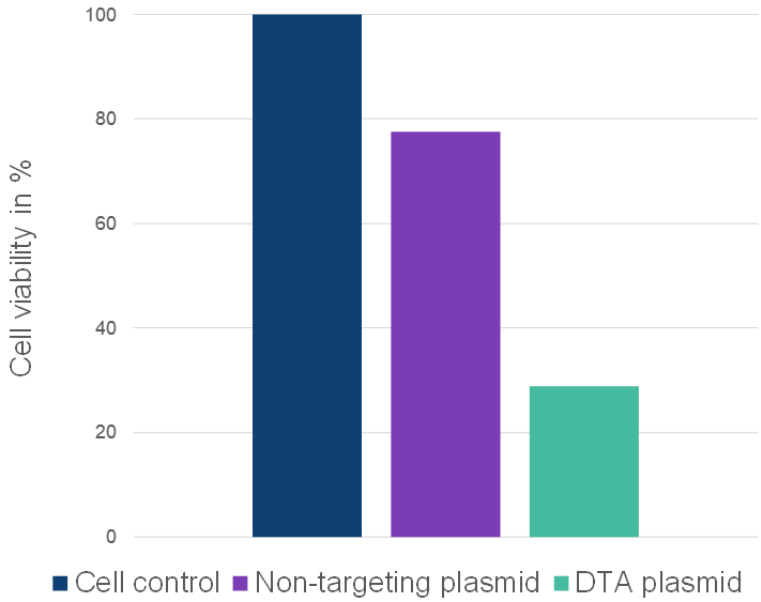


Figure 28. MTT assay in BT12 cells in suspension for exosome-based therapy. HEK293 exosomes. Use of 10 kDa centrifugal filter tubes. One inoculation with 3 µg of DTS-pIL13RA2s-DTA.

Signs of apoptosis were detected in all cells treated with the exosome mediated DTA gene therapy and more cells were detected in the control cells. The red fluorescence signal in the control cells indicated a successful uptake of the mCherry transfected exosomes by the glioblastoma cells. The MTT assay showed a substantially decreased viability of the cells treated with the exosomes containing the DTS-pIL13RA2s-DTA plasmids compared to that of the control cells (Figure 26 and 28). The viability of the DTA exosome therapy ranged from 29% to 38% and therefore, resulted in a substantial difference compared to the control cells in each experiment. These results demonstrate the efficacy of the exosome mediated DTA therapy. Also, in this case we cloned the DTS-pIL13RA2s-DTA plasmid into the miniplasmid pUCmu to increase the transfection efficiency by decreasing the plasmid size. We also subsequently studied the efficacy of the combination therapy using exosomes containing both the pUCmu-DTS-pIL13RA2s-DTA and pUCmu-DTS-hpmir21-dGzmB + NLS 3x plasmids.

3.4.6 Combination therapy with Granzyme B and Diphtheria toxin fragment A indicates synergistic effect on glioblastoma cells

We treated the BT12 cells with a combination of exosomes containing both the pUCmu-DTS-pIL13RA2s-DTA and pUCmu-DTS-hpmir21-dGzmB + NLS 3x targeting plasmids. Untreated cells, cells incubated with PBS and three additional conditions

were used as controls. In these additional controls, one of the components (exosomes, plasmid and transfection reagent Fugene HD) was each left out. Each treatment was performed in triplicate. Cell viability was determined by using the MTT assay 96 hours after the inoculation of the transfected exosomes. The MTT assay showed none or only minor effect on cell viability with any of the control conditions, while the viability of the cells treated with the combination therapy with Granzyme B and Diphtheria toxin fragment A was reduced to about 75% (Figure 29).

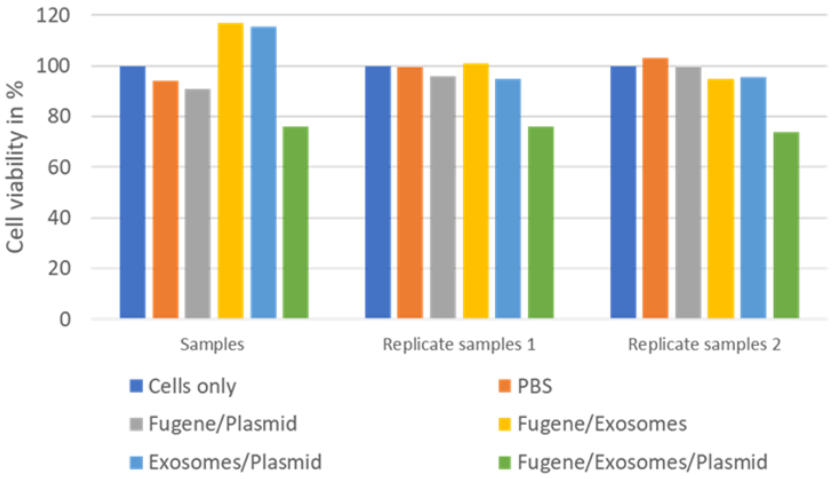


Figure 29. MTT assay in BT12 cells in adherent condition for exosome-based therapy. HEK293 exosomes. Use of 50 kDa centrifugal filter tubes. One inoculation with 3 µg of pUCmu-DTS-hpmir21-dGzmB + NLS 3x and 3 µg of pUCmu-DTS-pIL13RA2s-DTA in combination therapy.

This experiment was repeated to validate the results. The second experiment showed very similar results than the first one with one exception. The control containing the transfection reagent Fugene HD and exosomes with left out plasmids decreased cell viability by approximately 27% (Figure 30). The viability of cells treated with the combination therapy was decreased by around 43% (Figure 30). While there was a deviation from the first experiment, the difference between the viability of cells treated with the combination therapy and the lowest cell viability of any of the controls remained in between 15 – 20% indicating that the efficiency of the gene therapy did not change.

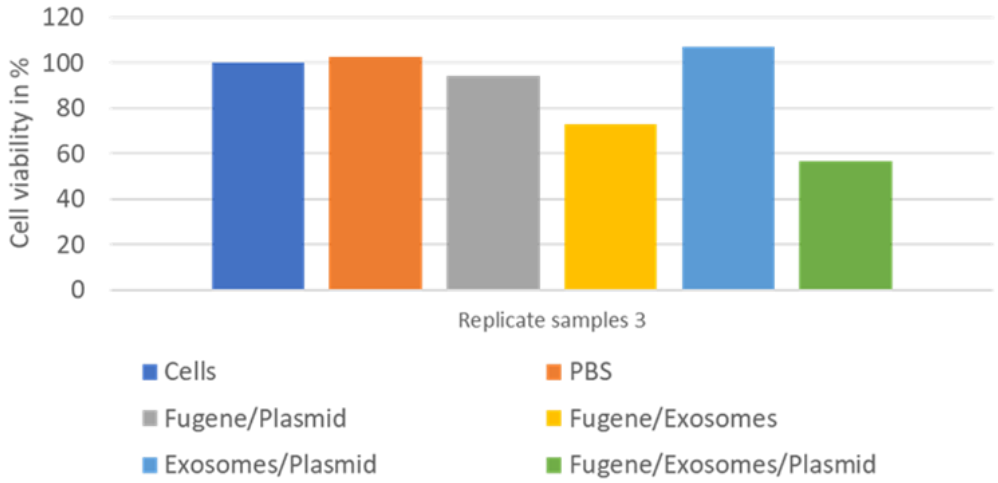


Figure 30. MTT assay in BT12 cells in adherent condition for exosome-based therapy. HEK293 exosomes. Use of 50 kDa centrifugal filter tubes. One inoculation with 3 µg of pUCmu-DTS-hpmir21-dGzmB + NLS 3x and 3 µg of pUCmu-DTS-pIL13RA2s-DTA in combination therapy.

The single treatment of targeting plasmid encoding GzmB showed a cell viability decrease of 11 to 19% compared to the control condition after one exosome inoculation. Meanwhile, the combination treatment in cells decreased the cell viability by 29,4% after one inoculation, indicating a synergistic effect of the targeting plasmids encoding GzmB and DTA.

The average of the MTT assays for the combination therapy was calculated and is displayed in Figure 31. All in all, results clearly demonstrate that the combination therapy resulted in a decreased cell viability of 70.6% with one inoculation. The control conditions showed stable results with only minor decreases in cell viability, hereby proving that neither any potential external plasmid outside of the exosomes nor the toxicity of the transfection reagent Fugene HD or any passive diffusion of the plasmids into the exosomes was responsible for the decrease in cell viability in the gene therapy condition (Figure 31).

The data obtained from the experiments was analyzed and an ANOVA test was performed to test for the significance of the data. The ANOVA test results are shown in Appendix C. The ANOVA test and the t-test both showed that the data for the combination therapy was significant when compared to the control conditions. Overall, it was proven that the set-up with exosomes isolated from HEK293FT cells and the combination therapy proved to be successful in glioblastoma cells after one inoculation.

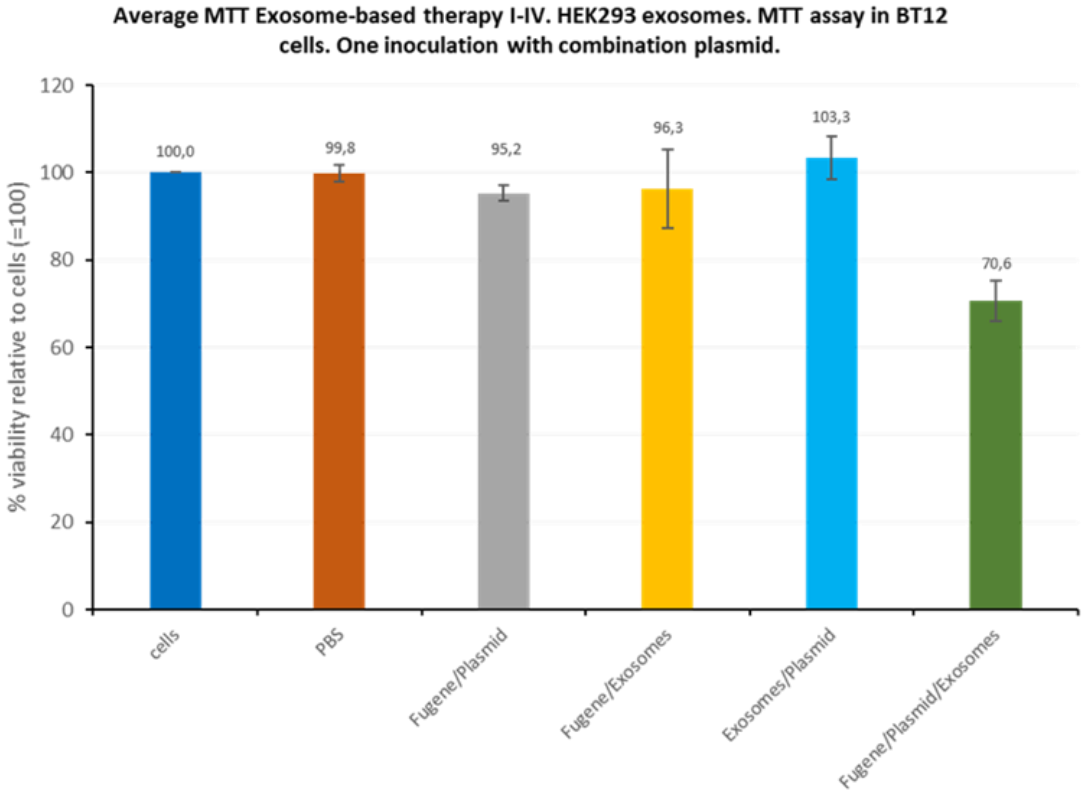


Figure 31. Average of MTT assays for exosome-based gene therapy I - IV in BT12 cells in adherent condition. HEK293 exosomes. Use of 50 kDa centrifugal filter tubes. One inoculation with 3 µg of pUCmu-DTS-hpmir21-dGzmB + NLS 3x and 3 µg of pUCmu-DTS-pIL13RA2s-DTA in combination therapy.

4 Discussion

4.1 Limited efficiency of gene therapy targeting common alterations in glioblastoma cells due to elevated plasmid size

The plasmids used to inhibit the expression of the oncogene EGFRvIII and overexpress the tumor suppressor genes TP53 and PTEN were utilized with the goal of targeting the most common alterations in glioblastoma cells and thereby decreasing the cell proliferation and increasing apoptotic cell death. All the plasmids were approximately 5700 bp and therefore significantly larger than the RNAi that has been delivered by exosomes prior to this project (Lozada-Delgado et al., 2017). Examples of the research done with exosomes is a study introducing siRNAs in exosomes derived from dendritic cells by electroporation with the aim of reducing the gene expression of a therapeutic target in Alzheimer´s disease. The results showed approximately 60% reduction in mRNA and protein expression of the therapeutic target (Sancho-Albero et al., 2020). A different study focused on isolating exosomes from immature dendritic cells, which were derived from the bone marrow of mice and subsequently the exosomes were used to deliver siRNA *in vitro* and *in vivo* in a targeted manner (Jiang & Gao, 2017). Additionally, it was demonstrated that modifying the membrane of exosomes with the aim of enhancing the targeting efficiency is possible. A protein targeting neurons was expressed on the surface of exosomes and following the intravenous injection of the exosomes in mice, the exosomes succeeded in reducing specific gene expression in the brain (Jiang & Gao, 2017). An additional study proved that exosomes can deliver exogenous miRNAs and thereby elicit a biological response in recipient cells (EL Andaloussi et al., 2013). These findings demonstrate the potential of loading RNA into exosomes with the aim of developing a targeted therapy in cancer or neurologic disorders, such as Alzheimer disease. However, these studies also display the current focus on the exosome uptake of smaller molecules, such as RNA, while there is a lack of research for exosomes taking up plasmid DNA. Due to the limited size of exosomes and the size-dependent transfection efficiency, a reduction in the transfection efficiency was anticipated when using larger plasmid DNA. Following the first inoculation of the transfected exosomes targeting common genetic alterations in glioblastoma cells, only approximately 20 –

30% of cells treated with the targeting plasmids showed signs of apoptosis and decreased proliferation, while an equal number of cells treated with the control plasmids encoding fluorescent proteins, successfully showed fluorescent signal. As a result of this outcome, a second inoculation was performed, which only slightly increased the visible apoptotic and fluorescent signal in the cells. After a third and final inoculation of transfected exosomes into the cells, the targeted cells showed strong apoptotic signal and the fluorescent signal in the control cells increased to approximately 60%. However, three inoculations required a large number of transfected exosomes, that were inoculated in a single well of a 96-well plate, hereby making a potential transfer to *in vivo* experiments unlikely. Consequently, the decision was made to utilize cytotoxins, which are known to induce apoptosis in cancer cells with the aim of increasing the efficiency of the treatment with a smaller number of inoculations.

4.2 Cloning the targeting plasmids containing cytotoxins into miniplasmid pUCmu increased the transfection efficiency

The treatment was started with the targeting plasmid hpmir21-dGzmB in the patient-derived glioblastoma cell line BT12, resulting in cell death in the targeted cells after two inoculations during imaging. Consequently, to enhance the efficiency of the GzmB plasmid and thereby the treatment outcome, the NLS and DTS sequences were added to the GzmB plasmid, resulting in an increased uptake of the GzmB plasmid in the nucleus of the target cells. The MTT assay following the treatment showed an 80% decreased cell viability for the gene therapy treated cells after one inoculation, thereby increasing the treatment efficiency compared to the gene therapy targeting the glioblastoma cell alterations. Furthermore, it was decided to reduce the plasmids in size due to the size-dependent transfection efficiency (Hardee et al., 2017). After cloning the GzmB plasmid into the miniplasmid pUCmu, the resulting plasmid construct pUCmu-DTS-hpmir21-dGzmB + NLS 3x was decreased in size by 600 bp to a total size of 3388 bp. Following the molecular cloning, another reduction of the cell viability in the gene therapy set-up could be seen. In total, cell viability decreased by 93,1% in the treated condition after one inoculation, thus displaying the important correlation between the construct size and the transfection efficiency.

As a result of this promising outcome, the DTA plasmid was cloned into the miniplasmid pUCmu as well, with the size of the final plasmid construct pUCmu-DTS-pIL13RA2s-DTA being reduced to 3828 bp by removing approximately 600 bp. The DTA plasmid was used due to its proven inhibition of the protein synthesis, resulting in apoptosis in cancer cells (Shafiee et al., 2019). While a plasmid-based expression of DTA in exosomes has not been shown so far, DTA has been used in a variety of studies.

A study researching gene therapy for hepatocellular carcinoma assessed the efficiency of the DTA-expressing plasmid *in vivo*, by hydrodynamically delivering the naked plasmid DNA to the mouse liver. The results indicate the inhibition of the protein synthesis, apoptotic changes and the antitumor effect of the DTA expression in the mouse model (Kamimura et al., 2020). For the therapy of transitional cell carcinoma (TCC) bladder carcinoma, a strategy was developed resulting in the DTA expression driven by the IGF2 P3 and P4 human promoters, which have been shown to be particularly active in various human cancers. The results showed a 70% inhibited growth rate of the developed tumors in the treated human carcinoma cell lines following intratumorally injection (Ayesh et al., 2003). In a recent study, DTA was used for the delivery of siRNAs for the first time with the toxin-based delivery of siRNA resulting in gene downregulation in patient-derived glioblastoma cells (Arnold et al., 2020). Another study focused on the development of a new therapeutic approach in ovarian cancer cells with the aim of targeting the DTA expression under the control of H19 regulatory sequences. H19 RNA has been detected in abundance in ovarian cancer and other human cancer tissues, however it is almost undetectable in healthy tissue. DTA-H19 was injected intratumorally into developed tumors and resulted in a 40% inhibition of tumor growth (Mizrahi et al., 2009). These findings display the toxicity of the Diphtheria toxin and its efficiency in the therapy of varying cancer types. Our gene therapy set-up with DTA in exosomes resulted in cell viability ranging from 29% to 38% when compared to the control cells and thereby succeeded in demonstrating the efficacy of the exosome mediated DTA therapy.

4.3 Induction of the death of target cells was due to the plasmids outside of the exosomes

To study the potential effect of external plasmids on the target cells, the DNAse I enzyme was used in the process of inoculation of the transfected exosomes into the

cells. Here, it was discovered that the cell viability decreases only by approximately 15 to 20% in the targeted cells compared to the control cells, which was a reduction from the 93,1% without the DNAse I addition. Hence, these findings were a strong indicator that the apoptotic effect in the glioblastoma cells was not only due to the transfected exosomes but also external plasmids, that had not been taken up by the exosomes.

The results of this project indicate that the DNAse I enzyme was successful in removing external plasmid, since the cells treated with the gene therapy and without the addition of exosomes did not show any decrease in cell viability at all. This suggests that the 20% decrease in cell viability in the target cells are solely due to the exosome-based gene therapy.

A study revealed that loading short linear double stranded DNA (dsDNA) into extracellular vesicles by electroporation with the addition of DNAse I enzyme resulted in the degradation of the majority of DNA, associated with the extracellular vesicles. However, on average hundreds of copies of DNA per extracellular vesicle still remained (Lamichhane et al., 2015). These findings would explain the difference in cell viability decrease that was seen after the addition of the DNAse I enzyme and further encourage that the decrease in cell viability in the treated cells with the addition of the DNAse I enzyme occurred due to the remaining exosome-based gene therapy. However, it has to be noted that the DNA loading method into the exosomes differed and the plasmid DNA used in this project had an average size of 3500 bp, while the study used a 250 bp dsDNA.

4.4 Potential toxicity of chemical transfection reagent Fugene HD on glioblastoma cells

While optimizing the varying conditions in glioblastoma cells to determine the causes for the decreased cell viability, it was observed that while the majority of the control cells showed reproducible results, the transfection reagent Fugene HD and exosomes displayed variable results. These variations were also noted in experiments that included exosomes and plasmid. Since the transfection reagent seemed likely to be the reason for this non-specific behavior, the ratio of Fugene-Plasmid was reduced from a 1:4 to a 1:3 ratio, which resulted in a reduction of the toxicity related to the Fugene HD but failed to completely erase the variations in cell viability over the courses of several experiments. Studies have shown that commercial transfection reagents can be used to introduce siRNA or other genetic material into exosomes but the

electroporation method resulted in a higher efficiency of transfected exosomes compared to the transfection method (Jiang & Gao, 2017). As a consequence of the comparably low efficiency of the transfection method, we researched alternative methods for loading DNA into exosomes.

Recent studies showed the varying exogenous techniques for loading plasmids into extracellular vesicles, such as transfection, electroporation, and sonication (Orefice, 2020). The transfection method used in this study is simple to use and delivers a good plasmid DNA concentration. However, the disadvantages include that the transfection reagent could get partly hooked and the vesicle membranes might show deformations. Another disadvantage is the poor efficacy to transfect cells grown in suspension. Therefore, it was decided to use the alternative plasmid DNA loading method, electroporation, which comes with several advantages. These include loading of larger plasmid DNA and transfection of hard-to-transfect cells. Further, electroporation is quite versatile and results in direct uptake of the plasmid into the cells. While these advantages are beneficial to the experiments, there are also disadvantages that are connected to the electroporation, such as the potential damage of the cells and the possibility that the electroporation might result in an aggregation of the extracellular vesicles and thus alter the morphological characteristics of the exosomes (Orefice, 2020). A third alternative method would be sonication, which shows an increased loading efficiency and no chemical reagents are used in the process. However, during sonication the membrane could potentially get deformed through overheating and this method is only applicable to smaller sized plasmid DNA (Orefice, 2020). Hence, these limitations should be regarded, when selecting the transfection method.

Alternative methods to upload plasmid DNA into exosomes have been tested in the lab, such as electroporation, however, Fugene-HD has shown to be the most effective. The drawbacks of the electroporation method might be the reason, why electroporation has failed to show an increased efficiency compared to the transfection method with Fugene HD. In addition, studies have shown that electroporation has been successful for loading smaller molecules, such as siRNAs into extracellular vesicles (Lamichhane et al., 2015). However, loading DNA into exosomes has been associated with limitations due to the increased size and therefore primarily transfection-based approaches have been used so far (Lamichhane et al., 2015). These findings are

consistent with our research and therefore work is ongoing to minimize the toxic effects of the transfection reagent on cell viability.

4.5 OP9 cell derived exosomes are more efficient in cell killing than the HEK293FT cell derived exosomes

While the set-up of the exosome-based gene therapy was conducted using the HEK293FT cells as the source of the exosomes, one experiment was performed with virus transduced murine stem cell OP9 derived exosomes to study the efficiency of these delivery vesicles. In theory, these exosomes should result in a higher efficacy due to the engineered expression of the homing peptide iRGD on the surface of the exosomes. The homing peptide iRGD results in an improved targeting of the cancer cells and a better uptake of the exosomes into the glioblastoma cells, thereby increasing the efficiency of the gene therapy on the target cells.

The results showed a decrease in cell viability in both control and treated cells, which differed from the results obtained with the HEK293 derived exosomes, which did not show any or only a minor decrease in viability of control cells, while the treated cells showed reproducible results with 15 - 20% reduction in cell viability. In case of the OP9 derived exosomes, the viability of control cells decreased by approximately 40% while the cell viability of treated cells decreased by approximately 55%. Thus, the 15% increased cell death of treated cells compared to the control cells is similar to the results obtained using the HEK293FT derived exosomes. So far, there is no clear explanation for the strong decrease in cell viability in the control cells using the OP9 derived exosomes, but further experiments including the OP9 derived exosomes should provide insight into the potential mechanisms behind this.

4.6 Combination therapy results in the highest efficacy in glioblastoma cells

The targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x showed a reproducible 15 – 20% decrease in cell viability after one inoculation in the glioblastoma cell line BT12, which had been proven to be due to the exosome-based gene therapy. The combination therapy, resulting in the GzmB and DTA expression in the target cells demonstrated a 29,4% decrease in cell viability in the treated cells, while none or only minor effect was detected in the control cells. Even though the efficiency of the

chemical transfection utilizing the transfection reagent Fugene HD is generally quite low, the efficiency of the combination therapy was proven to be significant in this set-up. Thus, the combination therapy resulted in an additional 10 to 15% decrease in the viability of target cells and therefore it was the most efficient approach for the gene therapy in this study.

At the moment, no in-depth research is available for the use of exogenous plasmid DNA in extracellular vesicles (Orefice, 2020). Therefore, there are no publications concerning this strategy and the combination plasmid-based therapy in exosomes displays a novel set-up for the use of plasmid DNA in exosomes.

4.7 Advantages of exosome-based gene therapy utilizing targeting plasmids in glioblastoma cells

Several advantages are connected to the use of exosomes as the carriers for the gene therapy in glioblastoma cells. These include their availability, the decreased probability of immune reactions, the option of repeated administration of exosomes and their cost-effectiveness compared to the virus-derived vectors (Orefice, 2020). Thus, using exosomes as shuttles to transfer gene therapy to the cancer cells results in a decreased risk of immune reactions and toxicity and therefore increases the safety of the treatment. However, research is still limited when it comes to the ability of exosomes to take up plasmid DNA due to the limited exosomes size (Orefice, 2020). So far, double stranded DNA oligonucleotides such as plasmids have not been used for an exosome-based gene therapy since an increase of the size would negatively influence the transfection efficiency. By cloning the plasmid constructs into the miniplasmid pUCmu, the plasmid size was reduced, making it possible to transfect plasmids into exosomes and still perform an efficient transfection.

This project focused on a plasmid-based gene therapy due to their numerous advantages in the regulation of gene expression. Exosomes have the ability to target other cell types in addition to glioblastoma cells and the combination gene therapy, which was used in this project is nonspecific, allowing targeting of different cell types. However, the uptake of exosomes might vary for different cancer types, since the mechanisms used for the exosome uptake are dependent on the recipient cells (Horibe et al., 2018). Due to the use of plasmids in this project, a conditional expression was possible, which is not the case when RNA is used as the gene therapy. Conditional

expression is a vital concept for the safety of the plasmid-based gene therapies in exosomes by minimizing the side effects and increasing the safety of this set-up. This is achieved by using promoters that are particularly active and thereby show a conditional expression in cancer cells. Therefore, healthy cells, targeted by the transfected exosomes should not express the therapy and should not be damaged. Glioblastoma cells, on the other side have active promoters, resulting in expression of the therapy when targeted by the transfected exosomes creating a safe approach for the *in vivo* preclinical studies and future possible applications in patients.

Furthermore, using plasmids for the gene therapy enabled the finetuning of the plasmid expression by addition of enhancing sequences, such as the DNA translocation sequence (DTS) and the nuclear localization sequence (NLS), which were added to the Granzyme B plasmid to enhance the gene transcription and the uptake into the nucleus, where the damage took place. Additionally, control plasmids could be constructed, carrying sequences for fluorescent proteins, which was used to study the uptake of the transfected exosomes into the target cells. Overall, the possibility of finetuning and regulating the conditional expression are the main advantages of using plasmids in the exosome-based gene therapy.

4.8 Potential next steps in the research project

The efficiency of the combination therapy could potentially be increased by further optimizing the components of the set-up exosome-based gene therapy, such as the transfection reagent Fugene HD and the enzyme DNAse I. Determining a set amount of the transfection reagent Fugene HD could potentially lead to a reduction of the unspecific toxicity. In addition, the optimization of the DNAse I enzyme amount needed to remove the plasmid outside of the exosomes, while not damaging the target cells, might improve the efficacy of the combination therapy. To achieve this, an experiment could be set up, titrating the amount of DNAse in a control condition without exosomes and a treated condition with exosomes, followed by an imaging check-up of the cells and an MTT assay to confirm the results.

Additionally, the number of exosomes added for one inoculation and varying plasmid amounts could be studied, to define the optimal conditions for the exosome-based gene therapy set-up.

Furthermore, the combination therapy resulted in a significant decrease in cell viability in the treated cells after one inoculation, thereby entailing a potential increase of the treatment efficiency following additional inoculations. Consequently, the number of inoculations needs to be increased to study the efficiency of several inoculations. The combination therapy was only studied for one inoculation, which led to a decrease in cell variability of approximately 30%. Nevertheless, more inoculations would probably be required to result in at least 70% decrease in cell viability of the target cells. To achieve that, either two inoculations could be performed over the span of eight days, or two inoculations could be carried out at the same time point with an endpoint of the experiment after four days. In case this approach fails to improve the efficacy, a novel gene therapy with plasmids encoding different apoptosis inducing proteins could be studied in this experimental set-up.

After optimizing the experimental components and increasing the number of inoculations, the subsequent step would consist of repeating the set-up in other patient-derived glioblastoma cells.

Following the successful efficacy of the exosome-based gene therapy in at least two different glioblastoma cell lines, the next step in the project would include setting up the therapy for exosomes isolated from the virus transfected mesenchymal murine progenitor OP9 cell line.

While exosomes carry many receptors, due to the virus transduction, the exosomes isolated from the OP9 cells were equipped with an additional homing peptide receptor, the iRGD peptide (Yin et al., 2017). This iRGD homing peptide on the surface of the exosomes allows specific targeting of cancer cells and therefore should enhance the targeting of the glioblastoma cells (Yin et al., 2017). The enhanced targeting was not seen in the results, since the decrease in cell viability was similar to the decrease shown by HEK293FT derived exosomes. However, the results showed that the OP9 derived exosomes resulted in a strong toxicity in control and treated cells, which might be a reason for the lack of enhanced targeting efficiency. With additional research and further optimization of the OP9 derived exosomes, this problem could potentially be resolved.

Subsequently, the exosome-based gene therapy could be tested in *in vivo* preclinical models of glioblastoma to assess the efficacy.

4.9 Future opportunities

Glioblastoma continues to show a poor prognosis associated to low survival rates, which can be connected to its heterogenous histology, invasiveness and recurrent nature (Bălașa et al., 2020), along with a resistance to the conventional therapy approaches (Tang et al., 2019). Though the treatment for glioblastoma was continually improved in recent years, it is not possible to cure this cancer type yet (Hanif et al., 2017), with the therapy being mostly palliative (Kesari, 2011). In recent years, the increasing understanding behind the molecular mechanisms of glioblastoma development and progression has opened the door to new personalized treatment approaches (Bălașa et al., 2020), which are urgently needed.

So far, mRNA-based therapeutic models seem promising due to their many advantages, such as simple manipulation, fast expression and adaptive nature without mutagenesis, thereby being an optimal candidate for addressing the complex and variable nature of glioblastoma (Tang et al., 2019). Viruses have been used as one of the main delivery vehicles for the gene therapy, but in recent years, other alternative carriers have started to emerge (Kwiatkowska et al., 2013). Exosomes have shown many valuable advantages when compared to virus vectors and thus have started to become a promising new research subject for the delivery of gene therapies in cancer.

This study focused on a plasmid-based gene therapy in exosomes, which has not been used in therapeutic research in glioblastoma yet, but nevertheless facilitates conditional expression and finetuning of plasmids, thereby showing an important advantage compared to the mRNA-based therapeutic models. By setting up a plasmid-based gene therapy in exosomes for glioblastoma cells, the concept of this potentially new therapeutic model was proven, which could be further researched as a novel targeted therapy approach in glioblastoma cells.

Overall, this research project has shown a successful proof-of-concept by constructing a working plasmid-based gene therapy, delivered by exosomes. The exosomes then transfer these plasmids into target cells, decreasing the cell viability. Since the proof of principle is completed, the next step requires additional studying, finetuning and further research to receive the maximum efficacy in combination with minimal toxicity in the glioblastoma cells.

5 Acknowledgments

To Pirjo: Thank you for giving me the opportunity to conduct my master thesis in such an interesting research area in your lab and for your constant support and joyful lab atmosphere. The past year has been such a great experience full of wonderful memories and I am fully aware that I couldn't have picked a better lab for my thesis.

To Hector: Thank you for taking me as your master student last year and giving me the chance to be a part of this interesting project, even though my experience with exosomes was limited. I learned so much from you during this year and I am still amazed at how patient and supportive you were. This last year was so much fun and you and JK were such a big part of that. Thank you so much for being the best supervisor any student could ask for!

To JK: I really appreciate how much I learned from you about science, pipettes and western blots and I especially value how many times you made the effort to support me. It meant a lot to me, when you helped me with my thesis plan back in August as well as when you attended my thesis defense, even though you were already occupied. You were an amazing supervisor and a big part of our little lab family with Hector and Sara. Thank you for all the big and small moments with you, that will always make me smile when I think of them!

To Sara: You have been a constant in my life for the duration of my master´s thesis and you made this time a million times better. Thank you for being by my side for so many exosome isolations (for which you did an uncountable amount of column washes) and all of our conversations in and out of the lab and for making me laugh, even when I was tired. Just seeing you in the morning, when I came to the lab made my day and I know that our friendship will continue long after we both submitted our thesis. You are a truly amazing friend and I am so thankful to this group for bringing us together. We share so many great memories working alongside each other day and night in the lab and we always pushed each other to the limits. You were also the first person to talk to when I wanted to experience the Finnish way of life including Finnish recipes. I cannot wait for all the wonderful experiences still to come.

To the Laakkonen Lab members: Thank you Pauliina, Vadim, Rym, Onerva and Abi for your helpful advice during the lab meetings and for being such great supportive colleagues in the lab. A special thank you to Vadim for teaching me new skills with

mice after receiving my animal license and to Abi for brightening up many lab days by joking around with us but also for sharing a lot of valuable advice for our future. I truly enjoyed the time in the lab with you and I hope, that at some point once the pandemic is over, we get to do something fun together outside of the lab.

To Tiina Immonen: Thank you for your constant support especially during my first few weeks at the University of Helsinki. I really valued all your helpful advice.

To my TRANSMED colleagues: Thank you for all of the great times we had together, ranging from crayfish parties over kotiapprot to many memorable party nights! I never imagined to be part of a study program, in which everybody was so much fun to be around and even long lectures were a great thing, because it meant to spend time with all of you. Choosing this program has been one of the best choices I have ever made and meeting all of you is a big reason for that!

To my parents Silke & Dursun: I know that it was tough for you to let me move to a different country for two more years, after I had already spent my bachelor in Potsdam, many miles from home. It means the world to me, that even though my decision led to me missing many events and important moments, you still supported me with all you could give. If I am sure of one thing, it´s that you are proud of what I have achieved these past years and that you truly believe in me and for this, I wanted to say thank you to both of you! Especially a big thanks to my mum for always reminding me, that Germany has better weather and more sunlight. That really helped while trying to survive the Finnish winter. Ich liebe euch beide sehr! Danke für alles!

To my dearest twin sister Vanessa: Thank you for always being by my side, for being my greatest cheerleader and for your unwavering belief that I will always exceed in life, no matter what I do. You are the one, I can always talk to and you manage to make me laugh, even after the longest and most exhausting days. I know I wouldn´t be where I am without you. You are such a big part of who I am and witnessing your discipline in your law studies makes me want to be better in life and encourages me to work harder every day. Thank you for our constant Skype calls, which always last for hours and make me feel like you are right there beside me. Being apart from you for these past 6 years has been incredibly hard and I look forward to the coming months and years with you, in which we will hopefully spend a lot of time by each other´s side. Love you 5000!

6 References

Addgene 2019, Generating Stable Cell Lines with Lentivirus, accessed 22 April 2021, <https://www.addgene.org/protocols/generating-stable-cell-lines/>

Alifieris, C., & Trafalis, D. T. (2015). Glioblastoma multiforme: Pathogenesis and treatment. In *Pharmacology and Therapeutics*. https://doi.org/10.1016/j.pharmthera.2015.05.005

Arnold, A. E., Smith, L. J., Beilhartz, G., Bahlmann, L. C., Jameson, E., Melnyk, R., & Shoichet, M. S. (2020). Attenuated diphtheria toxin mediates siRNA delivery. *Science Advances*. https://doi.org/10.1126/sciadv.aaz4848

Ayesh, B., Matouk, I., Ohana, P., Sughayer, M. A., Birman, T., Ayesh, S., Schneider, T., de Groot, N., & Hochberg, A. (2003). Inhibition of tumor growth by DT-A expressed under the control of IGF2 P3 and P4 promoter sequences. *Molecular Therapy*. https://doi.org/10.1016/S1525-0016(03)00056-X

Bălașa, A., Șerban, G., Chinezu, R., Hurghiș, C., Tămaș, F., & Manu, D. (2020). The involvement of exosomes in glioblastoma development, diagnosis, prognosis, and treatment. In *Brain Sciences*. https://doi.org/10.3390/brainsci10080553

Bastos, N., Ruivo, C. F., da Silva, S., & Melo, S. A. (2018). Exosomes in cancer: Use them or target them? In *Seminars in Cell and Developmental Biology*. https://doi.org/10.1016/j.semcdb.2017.08.009

Batash, R., Asna, N., Schaffer, P., Francis, N., & Schaffer, M. (2017). Glioblastoma Multiforme, Diagnosis and Treatment; Recent Literature Review. *Current Medicinal Chemistry*. https://doi.org/10.2174/0929867324666170516123206

Benitez, J. A., Ma, J., D'Antonio, M., Boyer, A., Camargo, M. F., Zanca, C., Kelly, S., Khodadadi-Jamayran, A., Jameson, N. M., Andersen, M., Miletic, H., Saberi, S., Frazer, K. A., Cavenee, W. K., & Furnari, F. B. (2017). PTEN regulates glioblastoma oncogenesis through chromatin-associated complexes of DAXX and histone H3.3. *Nature Communications*. https://doi.org/10.1038/ncomms15223

Chandramohan, V., Sampson, J. H., Pastan, I., & Bigner, D. D. (2012). Toxin-based targeted therapy for malignant brain tumors. In *Clinical and Developmental Immunology*. https://doi.org/10.1155/2012/480429

Clarke, J., Butowski, N., & Chang, S. (2010). Recent advances in therapy for glioblastoma. In *Archives of Neurology*. https://doi.org/10.1001/archneurol.2010.5

Davis, H. E., Morgan, J. R., & Yarmush, M. L. (2002). Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. *Biophysical Chemistry*. https://doi.org/10.1016/S0301-4622(02)00057-1

Davis, M. E. (2016). Glioblastoma: Overview of disease and treatment. *Clinical Journal of Oncology Nursing*. https://doi.org/10.1188/16.CJON.S1.2-8

De Toro, J., Herschlik, L., Waldner, C., & Mongini, C. (2015). Emerging roles of exosomes in normal and pathological conditions: New insights for diagnosis and

therapeutic applications. In *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2015.00203

EL Andaloussi, S., Lakhal, S., Mäger, I., & Wood, M. J. A. (2013). Exosomes for targeted siRNA delivery across biological barriers. In *Advanced Drug Delivery Reviews*. https://doi.org/10.1016/j.addr.2012.08.008

G. Castro, M., Candolfi, M., M. Kroeger, K., D. King, G., F. Curtin, J., Yagiz, K., Mineharu, Y., Assi, H., Wibowo, M., K.M. Ghulam Muhammad, A., Foulad, D., Puntel, M., & R. Lowenstein, P. (2011). Gene Therapy and Targeted Toxins for Glioma. *Current Gene Therapy*. https://doi.org/10.2174/156652311795684722

Gallego, O. (2015). Nonsurgical treatment of recurrent glioblastoma. In *Current Oncology*. https://doi.org/10.3747/co.22.2436

Gilligan, K. E., & Dwyer, R. M. (2017). Engineering exosomes for cancer therapy. In *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms18061122

Gourlay, J., Morokoff, A. P., Luwor, R. B., Zhu, H. J., Kaye, A. H., & Stylli, S. S. (2017). The emergent role of exosomes in glioma. In *Journal of Clinical Neuroscience*. https://doi.org/10.1016/j.jocn.2016.09.021

Grech, N., Dalli, T., Mizzi, S., Meilak, L., Calleja, N., & Zrinzo, A. (2020). Rising Incidence of Glioblastoma Multiforme in a Well-Defined Population. *Cureus*. https://doi.org/10.7759/cureus.8195

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. In *Cell*. https://doi.org/10.1016/S0092-8674(00)81683-9

Hanif, F., Muzaffar, K., Perveen, K., Malhi, S. M., & Simjee, S. U. (2017). Glioblastoma multiforme: A review of its epidemiology and pathogenesis through clinical presentation and treatment. In *Asian Pacific Journal of Cancer Prevention*. https://doi.org/10.22034/APJCP.2017.18.1.3

Hardee, C. L., Arévalo-Soliz, L. M., Hornstein, B. D., & Zechiedrich, L. (2017). Advances in non-viral DNA vectors for gene therapy. In *Genes*. https://doi.org/10.3390/genes8020065

Hehmann-Titt, G., Schiffer, S., Berges, N., Melmer, G., & Barth, S. (2013). Improving the Therapeutic Potential of Human Granzyme B for Targeted Cancer Therapy. *Antibodies*. https://doi.org/10.3390/antib2010019

Horibe, S., Tanahashi, T., Kawauchi, S., Murakami, Y., & Rikitake, Y. (2018). Mechanism of recipient cell-dependent differences in exosome uptake. *BMC Cancer*. https://doi.org/10.1186/s12885-017-3958-1

Jiang, X. C., & Gao, J. Q. (2017). Exosomes as novel bio-carriers for gene and drug delivery. In *International Journal of Pharmaceutics*. https://doi.org/10.1016/j.ijpharm.2017.02.038

Kalluri, R., & LeBleu, V. S. (2020). The biology, function, and biomedical applications of exosomes. In *Science* (Vol. 367, Issue 6478). https://doi.org/10.1126/science.aau6977

Kamimura, K., Yokoo, T., Abe, H., Sakai, N., Nagoya, T., Kobayashi, Y., Ohtsuka, M., Miura, H., Sakamaki, A., Kamimura, H., Miyamura, N., Nishina, H., & Terai, S.

(2020). Effect of diphtheria toxin-based gene therapy for hepatocellular carcinoma. *Cancers*. https://doi.org/10.3390/cancers12020472

Kesari, S. (2011). Understanding glioblastoma tumor biology: The potential to improve current diagnosis and treatments. *Seminars in Oncology*. https://doi.org/10.1053/j.seminoncol.2011.09.005

Kwiatkowska, A., Nandhu, M., Behera, P., Chiocca, E., & Viapiano, M. (2013). Strategies in Gene Therapy for Glioblastoma. *Cancers*. https://doi.org/10.3390/cancers5041271

Lamichhane, T. N., Raiker, R. S., & Jay, S. M. (2015). Exogenous DNA loading into extracellular vesicles via electroporation is size-dependent and enables limited gene delivery. *Molecular Pharmaceutics*. https://doi.org/10.1021/acs.molpharmaceut.5b00364

Le Joncour, V., Filppu, P., Hyvönen, M., Holopainen, M., Turunen, S. P., Sihto, H., Burghardt, I., Joensuu, H., Tynninen, O., Jääskeläinen, J., Weller, M., Lehti, K., Käkelä, R., & Laakkonen, P. (2019). Vulnerability of invasive glioblastoma cells to lysosomal membrane destabilization. *EMBO Molecular Medicine*. https://doi.org/10.15252/emmm.201809034

Li, X., Corbett, A. L., Taatizadeh, E., Tasnim, N., Little, J. P., Garnis, C., Daugaard, M., Guns, E., Hoorfar, M., & Li, I. T. S. (2019). Challenges and opportunities in exosome research—Perspectives from biology, engineering, and cancer therapy. *APL Bioengineering*. https://doi.org/10.1063/1.5087122

Lin, Y., Wu, J., Gu, W., Huang, Y., Tong, Z., Huang, L., & Tan, J. (2018). Exosome–Liposome Hybrid Nanoparticles Deliver CRISPR/Cas9 System in MSCs. *Advanced Science*. https://doi.org/10.1002/advs.201700611

Liu, C., & Su, C. (2019). Design strategies and application progress of therapeutic exosomes. In *Theranostics* (Vol. 9, Issue 4). https://doi.org/10.7150/thno.30853

Lozada-Delgado, E. L., Grafals-Ruiz, N., & Vivas-Mejía, P. E. (2017). RNA interference for glioblastoma therapy: Innovation ladder from the bench to clinical trials. In *Life Sciences*. https://doi.org/10.1016/j.lfs.2017.08.027

Martinez, R., Schackert, G., Yaya-Tur, R., Rojas-Marcos, I., Herman, J. G., & Esteller, M. (2007). Frequent hypermethylation of the DNA repair gene MGMT in long-term survivors of glioblastoma multiforme [1]. In *Journal of Neuro-Oncology*. https://doi.org/10.1007/s11060-006-9292-0

Mizrahi, A., Czerniak, A., Levy, T., Amiur, S., Gallula, J., Matouk, I., Abu-lail, R., Sorin, V., Birman, T., de Groot, N., Hochberg, A., & Ohana, P. (2009). Development of targeted therapy for ovarian cancer mediated by a plasmid expressing diphtheria toxin under the control of H19 regulatory sequences. *Journal of Translational Medicine*. https://doi.org/10.1186/1479-5876-7-69

Montano, N., Cenci, T., Martini, M., D'Alessandris, Q. G., Pelacchi, F., Ricci-Vitiani, L., Maira, G., de Maria, R., Larocca, L. M., & Pallini, R. (2011). Expression of EGFRvIII in glioblastoma: Prognostic significance revisited. *Neoplasia*. https://doi.org/10.1593/neo.111338

Mrugala, M. M. (2013). Advances and Challenges in the Treatment of Glioblastoma: A Clinician's Perspective. *Discovery Medicine*.

New England Biolabs 2021, Gibson Assembly®, accessed 8 February 2021, <https://international.neb.com/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/gibson-assembly>

New England Biolabs 2021, NEBuilder HiFi DNA Assembly Reaction Protocol, accessed 13 February 2021, <https://international.neb.com/protocols/2014/11/26/nebuilder-hifi-dna-assembly-reaction-protocol>

O'Loughlin, A., Woffindale, C., & Wood, M. (2012). Exosomes and the Emerging Field of Exosome-Based Gene Therapy. *Current Gene Therapy*, O'Loughlin, A., Woffindale, C., Wood, M. (2012). https://doi.org/10.2174/156652312802083594

Orefice, N. S. (2020). Development of new strategies using extracellular vesicles loaded with exogenous nucleic acid. In *Pharmaceutics*. https://doi.org/10.3390/pharmaceutics12080705

Padfield, E., Ellis, H. P., & Kurian, K. M. (2015). Current therapeutic advances targeting EGFR and EGFRvIII in glioblastoma. *Frontiers in Oncology*. https://doi.org/10.3389/fonc.2015.00005

Pullan, J. E., Confeld, M. I., Osborn, J. K., Kim, J., Sarkar, K., & Mallik, S. (2019). Exosomes as Drug Carriers for Cancer Therapy. *Molecular Pharmaceutics*. https://doi.org/10.1021/acs.molpharmaceut.9b00104

Rousalova, I., & Krepela, E. (2010). Granzyme B-induced apoptosis in cancer cells and its regulation (review). In *International Journal of Oncology*. https://doi.org/10.3892/ijo-00000788

Sancho-Albero, M., Medel-Martínez, A., & Martín-Duque, P. (2020). Use of exosomes as vectors to carry advanced therapies. *RSC Advances*. https://doi.org/10.1039/d0ra02414g

Shafiee, F., Aucoin, M. G., & Jahanian-Najafabadi, A. (2019). Targeted Diphtheria Toxin-Based Therapy: A Review Article. In *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2019.02340

Sharma, A., Khatun, Z., & Shiras, A. (2016). Tumor exosomes: Cellular postmen of cancer diagnosis and personalized therapy. In *Nanomedicine*. https://doi.org/10.2217/nnm.15.210

Srivastava, A., Filant, J., Moxley, K., Sood, A., McMeekin, S., & Ramesh, R. (2015). Exosomes: A Role for Naturally Occurring Nanovesicles in Cancer Growth, Diagnosis and Treatment. *Current Gene Therapy*. https://doi.org/10.2174/1566523214666141224100612

Sugahara, K. N., Teesalu, T., Karmali, P. P., Kotamraju, V. R., Agemy, L., Girard, O. M., Hanahan, D., Mattrey, R. F., & Ruoslahti, E. (2009). Tissue-Penetrating Delivery of Compounds and Nanoparticles into Tumors. *Cancer Cell*. https://doi.org/10.1016/j.ccr.2009.10.013

Sun, N. feng, Liu, Z. ao, Huang, W. bai, Tian, A. ling, & Hu, S. yuan. (2014). The research of nanoparticles as gene vector for tumor gene therapy. In *Critical Reviews in Oncology/Hematology*. https://doi.org/10.1016/j.critrevonc.2013.10.006

Tamimi, A. F., & Juweid, M. (2017). Epidemiology and Outcome of Glioblastoma. In *Glioblastoma*. https://doi.org/10.15586/codon.glioblastoma.2017.ch8

Tang, X., Zhang, S., Fu, R., Zhang, L., Huang, K., Peng, H., Dai, L., & Chen, Q. (2019). Therapeutic Prospects of mRNA-Based Gene Therapy for Glioblastoma. In *Frontiers in Oncology*. https://doi.org/10.3389/fonc.2019.01208

Wei, W., Ao, Q., Wang, X., Cao, Y., Liu, Y., Zheng, S. G., & Tian, X. (2021). Mesenchymal Stem Cell–Derived Exosomes: A Promising Biological Tool in Nanomedicine. In *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2020.590470

Wirsching, H. G., Galanis, E., & Weller, M. (2016). Glioblastoma. In *Handbook of Clinical Neurology*. https://doi.org/10.1016/B978-0-12-802997-8.00023-2

Yang, J. M., Schiapparelli, P., Nguyen, H. N., Igarashi, A., Zhang, Q., Abbadi, S., Amzel, L. M., Sesaki, H., Quinõnes-Hinojosa, A., & Iijima, M. (2017). Characterization of PTEN mutations in brain cancer reveals that pten mono-ubiquitination promotes protein stability and nuclear localization. *Oncogene*. https://doi.org/10.1038/onc.2016.493

Yin, H., Zhang, Q., Yang, J., Wang, H., Xu, J., & Zheng, J. (2017). IRGD as a tumor-penetrating peptide for cancer therapy (Review). In *Molecular Medicine Reports*. https://doi.org/10.3892/mmr.2017.6419

Zhang, J., Wei, H., Guo, X., Hu, M., Gao, F., Li, L., & Zhang, S. (2012). Functional verification of the diphtheria toxin A gene in a recombinant system. *Journal of Animal Science and Biotechnology*. https://doi.org/10.1186/2049-1891-3-29

Zhang, Y., Dube, C., Gibert, M., Cruickshanks, N., Wang, B., Coughlan, M., Yang, Y., Setiady, I., Deveau, C., Saoud, K., Grello, C., Oxford, M., Yuan, F., & Abounader, R. (2018). The p53 pathway in glioblastoma. In *Cancers*. https://doi.org/10.3390/cancers10090297

7 Appendices

7.1 Appendix A | Gel electrophoresis pictures

The gel electrophoresis pictures taken as a confirmation following the molecular cloning for the targeting and non-targeting plasmids are displayed in the Figures 32 – 35.

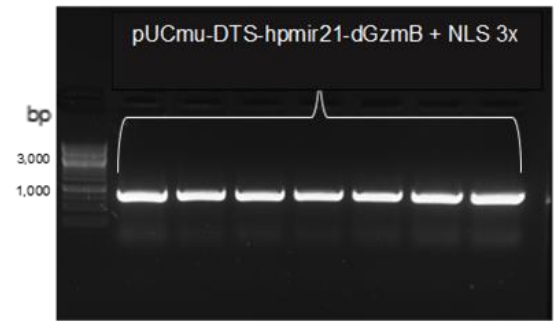


Figure 32. Gel electrophoresis picture for confirmation of molecular cloning for targeting plasmid pUCmu-DTS-hpmir21-dGzmB + NLS 3x with expected band length of 800 bp.

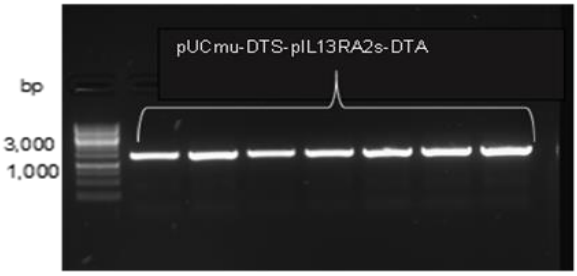


Figure 33. Gel electrophoresis picture for confirmation of molecular cloning for targeting plasmid pUCmu-DTS-pIL13RA2s-DTA with expected band length of 1443 bp.

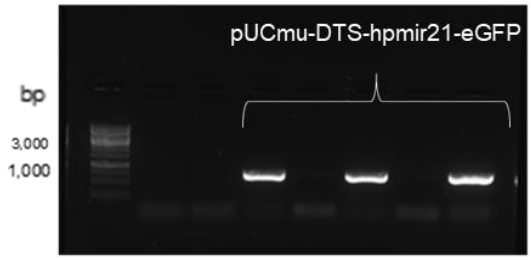


Figure 34. Gel electrophoresis picture for confirmation of molecular cloning for non-targeting plasmid pUCmu-DTS-hpmir21-eGFP with expected band length of 700 bp.

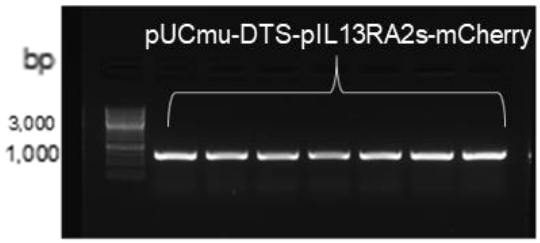


Figure 35. Gel electrophoresis picture for confirmation of molecular cloning for non-targeting plasmid pUCmu-DTS-pIL13RA2s-mCherry with expected band length of 700 bp.

7.2 Appendix B | Supplemental primer sequences

Table S1. Primer sequences used for plasmid construction.

Plasmid construct	Primer	Sequence	Annealing temperature [°C]	Size [bp]
pUCmu.dGzmB	pUCmu vector_fwd	aaggccgcgttgctggcgtttttccataggctccgccc	64	1611
	pUCmu vector_rev	aacttttctatacaaagttgggccatgggttaaccatatg		

	insert_fwd	caactttgtatagaaaagttgggtgtggaaag	67	1817
	insert_rev	aacgccagcaacgcggcc		
	GzmB Vect FW II	gcttcgagcagacatgataaga	63	803
	pUC ORI RV	gctctgctaatcctgttaccag		
pUCmu.GFP (dGzmB)	pUCmu vecGFP_fwd	gcatggacgagctgtacaagtaaacccagcttcttgtacaaag	62,4	
	pUCmu vecGFP_rev	tcctcgcccttgctcaccatggtggcagcctgcttttttg		
pUCmu.mCherry (insert)	mcherry Rev cloning	cttgtacagctcgtccatgcc	65	
	LSSMOranclon Fwd	atggtgagcaagggcgag		
pUCmu.GFP (insert)	LSSMOranclon Fwd	atggtgagcaagggcgag	63	
	pUC ORI RV	gctctgctaatcctgttaccag		
pUCmu.hpmir21-GFP + 3x NLS (dGzmB)	3x NLS fwd	gcatggacgagctgtacaagtccggactcagatctcgag	63	2744
	pUCmu vecGFP_rev	tcctcgcccttgctcaccatggtggcagcctgcttttttg		
	mcherry Rev cloning	cttgtacagctcgtccatgcc	65	717
	LSSMOranclon Fwd	atggtgagcaagggcgag		
	LSSMOranclon Fwd	atggtgagcaagggcgag	63	1652
	pUC ORI RV	gctctgctaatcctgttaccag		
pUCmu.DTS.pIL13RA2.DTA	pUCmu vector_fwd	aaggccgcgttgctggcgtttttccataggctccgccc	61	1704
	vector_rev	atggaggatcttagcagggatggttgctgactaattgagatg		
	pIL13RA2_fwd	tccctgctaagatcctcc	61	951
	pIL13RA2_rev	tacaaacttgaaaaacccttgtagctcctc		

	DTA_fwd	aagggtttttcaagtttgtacaaaaaagcaggctgcc	68	1233
	insert_rev	aacgccagcaacgcggcc		
	DTA check RV	aagcaccatcaccgaacc	61	1443
	pIL13RA2_fwd	tccctgctaagatcctcc		
pUCmu.enh.pIL13RA2.DTA	pIL13RA2_fwd	tccctgctaagatcctcc	62	2003
	vector_rev II	atggaggatcttagcagggacatggtaatagcgatgactaatacg		
pUCmu.CMV_enh.∆GzmB	PNL_Enh_Vect_fwd	aactttgtatagaaaagttgcatgcattagttattaatagtaatcaattac	59	3316
	pUCmu vector_rev II	caacttttctatacaaagttggg		
	CMV_enh_FW II	aactttgtatagaaaagttgcatgcattagttattaatagtaatcaattac	59	411
	CMV_enh_rev	aatttatgtactcggagctccatggtaatagcgatgactaatac		
	CMV_enh Check Fwd	TCCCATAGTAACGCCAATAGG	62	845
	GzmB RV Check	ACAGTGAGCAGCTGTCAGC		

Table S2. Primer sequences used for PCR expression check following plasmid construction.

Plasmid	Primer	Sequence	Annealing temperature [°C]	Size [bp]
GFP	LSSMOranclon Fwd	atggtgagcaagggcgag	65	717
	mcherry Rev cloning	cttgtacagctcgtccatgcc		
mCherry	LSSMOranclon Fwd	atggtgagcaagggcgag	64	385
	mcherry check Rev	gcttcaccttgtagatgaactcg		

DTA	DTA check FW	gtgagcaggaagctgttcg	63	469
	DTA check RV	aagcaccatcaccgaacc		
GzmB	GzmB check FW	cctgatacgagacgacttcg	62	509
	GzmB check RV	tcgtccataggagacaatgc		

7.3 Appendix C | Supplemental One-way and Post Hoc ANOVA test report in SPSS for average MTT Exosome-based therapy I – IV utilizing combination plasmid

Oneway

Notes

Output Created		17-FEB-2021 15:40:03
Comments		
Input	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	24
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics for each analysis are based on cases with no missing data for any variable in the analysis.
Syntax		ONEWAY MTT BY Groups /MISSING ANALYSIS.
Resources	Processor Time	00:00:00.02
	Elapsed Time	00:00:00.00

[DataSet1]

ANOVA

MTT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2838.837	5	567.767	6.293	.002
Within Groups	1623.892	18	90.216		
Total	4462.730	23			

```
ONEWAY MTT BY Groups
  /MISSING ANALYSIS
  /POSTHOC=TUKEY BONFERRONI ALPHA(0.05).
```

Oneway

Notes

Output Created		17-FEB-2021 15:42:51
Comments		
Input	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	24
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics for each analysis are based on cases with no missing data for any variable in the analysis.
Syntax		ONEWAY MTT BY Groups /MISSING ANALYSIS /POSTHOC=TUKEY BONFERRONI ALPHA(0.05).
Resources	Processor Time	00:00:00.02
	Elapsed Time	00:00:00.01

ANOVA

MTT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2838.837	5	567.767	6.293	.002
Within Groups	1623.892	18	90.216		
Total	4462.730	23			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: MTT

	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Tukey HSD	Cells	PBS	.175	6.716	1.000	-21.17	21.52
		Fugene/Plasmid	4.775	6.716	.978	-16.57	26.12
		Fugene/Exosomes	3.675	6.716	.993	-17.67	25.02
		Exosomes/Plasmid	-3.250	6.716	.996	-24.59	18.09
		Exosome/Plasmid/Fugene	29.400*	6.716	.004	8.06	50.74
	PBS	Cells	-.175	6.716	1.000	-21.52	21.17
		Fugene/Plasmid	4.600	6.716	.981	-16.74	25.94
		Fugene/Exosomes	3.500	6.716	.995	-17.84	24.84
		Exosomes/Plasmid	-3.425	6.716	.995	-24.77	17.92
		Exosome/Plasmid/Fugene	29.225*	6.716	.004	7.88	50.57
	Fugene/Plasmid	Cells	-4.775	6.716	.978	-26.12	16.57
		PBS	-4.600	6.716	.981	-25.94	16.74
		Fugene/Exosomes	-1.100	6.716	1.000	-22.44	20.24
		Exosomes/Plasmid	-8.025	6.716	.834	-29.37	13.32
		Exosome/Plasmid/Fugene	24.625*	6.716	.019	3.28	45.97
	Fugene/Exosomes	Cells	-3.675	6.716	.993	-25.02	17.67
		PBS	-3.500	6.716	.995	-24.84	17.84
		Fugene/Plasmid	1.100	6.716	1.000	-20.24	22.44
		Exosomes/Plasmid	-6.925	6.716	.901	-28.27	14.42
		Exosome/Plasmid/Fugene	25.725*	6.716	.013	4.38	47.07

	Exosomes/Plasmid	Cells	3.250	6.716	.996	-18.09	24.59
		PBS	3.425	6.716	.995	-17.92	24.77
		Fugene/Plasmid	8.025	6.716	.834	-13.32	29.37
		Fugene/Exosomes	6.925	6.716	.901	-14.42	28.27
		Exosome/Plasmid/Fugene	32.650*	6.716	.001	11.31	53.99
	Exosome/Plasmid/Fugene	Cells	-29.400*	6.716	.004	-50.74	-8.06
		PBS	-29.225*	6.716	.004	-50.57	-7.88
		Fugene/Plasmid	-24.625*	6.716	.019	-45.97	-3.28
		Fugene/Exosomes	-25.725*	6.716	.013	-47.07	-4.38
		Exosomes/Plasmid	-32.650*	6.716	.001	-53.99	-11.31
Bonferroni	Cells	PBS	.175	6.716	1.000	-22.53	22.88
		Fugene/Plasmid	4.775	6.716	1.000	-17.93	27.48
		Fugene/Exosomes	3.675	6.716	1.000	-19.03	26.38
		Exosomes/Plasmid	-3.250	6.716	1.000	-25.95	19.45
		Exosome/Plasmid/Fugene	29.400*	6.716	.005	6.70	52.10
	PBS	Cells	-.175	6.716	1.000	-22.88	22.53
		Fugene/Plasmid	4.600	6.716	1.000	-18.10	27.30
		Fugene/Exosomes	3.500	6.716	1.000	-19.20	26.20
		Exosomes/Plasmid	-3.425	6.716	1.000	-26.13	19.28
		Exosome/Plasmid/Fugene	29.225*	6.716	.006	6.52	51.93
	Fugene/Plasmid	Cells	-4.775	6.716	1.000	-27.48	17.93
		PBS	-4.600	6.716	1.000	-27.30	18.10
		Fugene/Exosomes	-1.100	6.716	1.000	-23.80	21.60
		Exosomes/Plasmid	-8.025	6.716	1.000	-30.73	14.68
		Exosome/Plasmid/Fugene	24.625*	6.716	.026	1.92	47.33
	Fugene/Exosomes	Cells	-3.675	6.716	1.000	-26.38	19.03
		PBS	-3.500	6.716	1.000	-26.20	19.20
		Fugene/Plasmid	1.100	6.716	1.000	-21.60	23.80
		Exosomes/Plasmid	-6.925	6.716	1.000	-29.63	15.78
		Exosome/Plasmid/Fugene	25.725*	6.716	.018	3.02	48.43
	Exosomes/Plasmid	Cells	3.250	6.716	1.000	-19.45	25.95
		PBS	3.425	6.716	1.000	-19.28	26.13
		Fugene/Plasmid	8.025	6.716	1.000	-14.68	30.73
		Fugene/Exosomes	6.925	6.716	1.000	-15.78	29.63
		Exosome/Plasmid/Fugene	32.650*	6.716	.002	9.95	55.35

	Exosome/Plasmid/Fugene	Cells	-29.400*	6.716	.005	-52.10	-6.70
		PBS	-29.225*	6.716	.006	-51.93	-6.52
		Fugene/Plasmid	-24.625*	6.716	.026	-47.33	-1.92
		Fugene/Exosomes	-25.725*	6.716	.018	-48.43	-3.02
		Exosomes/Plasmid	-32.650*	6.716	.002	-55.35	-9.95

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

MTT

			Subset for alpha = 0.05	
	Groups	N	1	2
Tukey HSD[a]	Exosome/Plasmid/Fugene	4	70.60	
	Fugene/Plasmid	4		95.23
	Fugene/Exosomes	4		96.32
	PBS	4		99.83
	Cells	4		100.00
	Exosomes/Plasmid	4		103.25
	Sig.		1.000	.834

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.